ASSAY OF POSSIBLE CARCINOGENS RELATED TO LUNG CANCER, WITH SPECIAL REFERENCE TO ATMOSPHERIC POLLUTION.

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Preface

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C O N T E N T S

I N T R O D U C T I O N.

The first intimation that an agent from the external environment might cause cancer of the lung came from the experience of miners in the Schneeberg region of Saxony, when the first diagnosis of lung cancer was made there in 1879, by Härting and Hesse. Among several hundred miners dying over a 37-year period prior to 1913, Arnstein found that cancer of the lungs accounted for about 40 per cent of all deaths. He identified the majority of lung tumours as squamous cell carcinomas.

Since that time occupational lung tumour hazards have been related to several industrial processes involving the use of chromates, nickel, asbestos, arsenic, as well as radioactive ores, among inorganic materials, and iso-propyl oil, petroleum oil and coal tar among organic materials (for Review see Hueper 1957). Limited populations were at risk, however.

Recent epidemiological studies strongly suggest that general population exposure to carcinogenic environmental agents is increasing. In support of this idea, the following facts are drawn up. A real, marked, and progressive increase in the incidence of pulmonary cancer has been observed in all industrialised or progressive countries during the last fifty years. The increase is unlikely to be due to changes in the hereditary composition of the population.

The increased incidence has occurred almost exclusively in the histological class of non-adenomatous tumours (largely squamous cell carcinomas) which are considered to be exogenous in origin, (Kreyberg 1954). The majority opinion holds that practically all such tumours arise from the basal cell layer of the bronchi, and some three-fourths from the epithelium of the larger bronchi (Liebow 1952). This topographical distribution when compared with that in other duct systems (urogenous and alimentary canal) points to their environmental origin, occupying as they do, sites of maximum exposure to hypothetical carcinogenic stimuli. The traffic pattern of air pollutants in the respiratory tract corresponds to the distribution pattern of respiratory cancers. This has prompted the evaluation of the etiological relevancy of possible carcinogens in atmospheric pollution.

It seemed reasonable to proceed from the known to the unknown. Thus, when the spectrum of occupational lung cancers, of more or less established etiology, is examined to ascertain whether the probable responsible factors have a more general distribution and thus hold etiological relevance in non-industrial lung cancer, the coal tar fume and petroleum oil cancer complexes would appear to meet the requirements. The carcinogenic agents presumed responsible for the disease in these instances, members of the polycyclic hydrocarbon class, have been detected (A) in the components of general atmospheric pollutions, e.g. the soots of incomplete combustion

processes, (B) in the products arising from the smoking of tobacco - an individualised type of air pollution. A higher incidence in urban over rural populations has been demonstrated by many investigators, e.g. Stocks (1952), and in an appraisal of the significance of this fact, Haenszel and Shimkin (1956) have noted "the urban-rural discrepancy, in our opinion, represents a real finding, and is a manifestation of multiple environmental factors in lung cancer." They concurred with the general consensus of statistical findings (Doll and Hill 1956; Hammond and Horn 1954) that cigarette smoking acts as the dominant etiological factor.

The present thesis records an investigation into some of the "multiple environmental factors" at the chemical level.

The chemical demonstration of agents in the environment which cause tumours in experimental animals does not "prove" a like relationship in the case of humans. When, however, a parallelism exists between epidemiological and laboratory findings, both assume greater significance. Such a parallelism has been claimed for the coal tar fume and soot cancer complex by Hueper (1957) and Kotin (1956) and for tobacco smoking, especially in the form of cigarettes, by Wynder and Graham (1950). But in evaluating such relationships other parameters must be taken into account. Kotin (1958a) observes that the identification of a carcinogen(s) for laboratory animals represents, essentially, the elucidation of but one link in cancer development, albeit the cardinal one. To minimise the importance of, or

to dismiss a weak experimental carcinogen in terms of human significance is insupportable. As a further parameter, the presence or absence of promoting agents in the atmosphere represents a small segment of the non-carcinogenic (or non-initiating) environmental milieu capable of modifying the carcinogenic response.

The evaluation of the role played by general atmospheric pollution in the causation of lung cancer during the past fifty years is greatly complicated as far as etiological factors are concerned. The atmosphere is dynamic with respect to concentration of presumed carcinogenic agents. At any one time the determinants involved are (a) source(s) of emission of the agents into the atmosphere, (b) season, (c) climate. Considering (b) and (c) first, Waller (1952) has shown that the 3,4-benzopyrene content of town air varied with the season, being higher in winter than in summer. The presence or absence of an "ozonising" atmosphere, the duration of sunlight, humidity, wind-flow, fog, each may affect the "carcinogenic" level of the atmosphere. A photochemically active atmosphere can result in the production of many new products. Falk et al. (1956) showed that the stability of individual aromatic hydrocarbons varied in the presence of an ozonising atmosphere in sunlight, - 3,4-benzopyrene was more resistant to destruction than anthracene, phenanthrene or "compound X". With regard to (a), Kreyberg's conclusion from statistical data compiled in Norway (Kreyberg 1954) that industrial effluents are not the essential factor

of that part of urban life which is responsible for the increase in lung cancer contrasts with the observation of Hueper (1953) which states that while the number of respiratory cancers which have been attributed to contact with industrial carcinogenic factors is relatively small, there is good reason for believing that the actual number of occupational respiratory cancers produced by an occupational exposure with them is considerably larger. Other sources of emission have altered considerably in the recent past. Kennaway and Kennaway (1947) remarked that the emission of soot from domestic fires and industrial concerns has fallen in recent years. In the United States, Hammond (1954) stressed the marked increase which occurred over the past thirty years in the amount of petrol and oil burned. These facts have led some workers, notably Kotin (1956) to put forward the following hypothesis: that the gradual shift from solid to liquid fuel throughout the world has led to the progressive enrichment of the atmosphere in compounds, presumed responsible for the tar fume and mineral oil mist or fume cancers of industry, in a physical state compatible with their retention in the lung and allowing of their access to the cells; that the resulting carcinogenic situation is largely responsible for the observed increased lung cancer mortality. The Los Angeles workers (Kotin et al. 1954-56) would appear to have stratified their case, from the laboratory level, by the successful production of skin tumours in mice by extracts of atmospheric,

petrol and diesel exhaust soots. The presence of 3,4-benzopyrene among other hydrocarbons was demonstrated in these extracts. Moreover an aliphatic fraction was prepared from the fuel combustion products which showed carcinogenic activity. The agents thought responsible here were epoxides, formed through the action of ozone on unsaturated hydrocarbons (Kotin and Falk 1955). Whether the ozonising atmosphere necessary for their formation exists in areas other than Los Angeles with its uniquely smoggy climate, is not known. A chief characteristic of the Los Angeles smog is its marked oxidising capacity (Haagen-Smit 1952) which may not prevail to the same extent elsewhere.

From the foregoing exposition, the following facts emerge; (a) that if general atmospheric pollution is an important parameter in the causation of lung cancers of the non-adenomatous histological group, the agents most likely responsible belong to the aromatic polycyclic hydrocarbon class of compounds, and (b) that the most probable source of general atmospheric enrichment with these compounds is the combustion products of liquid fuels in the form of vehicular exhausts. From this derives the rationale underlying the first part of the present thesis, which describes investigations into the composition of the aromatic hydrocarbon fraction of petrol and diesel exhaust soots. The particulate phase in both cases was chosen because the range of particle size is held to be compatible with maximum retention in the lung (Waller 1952). To

gauge their relative importance as foci of atmospheric pollution, a sample of general atmospheric soot was similarly investigated for comparison. In a publication (Lyons and Johnston 1957a) which described the essentials of this investigation, petrol exhaust soot received principal consideration. Though eclectic, this approach is held to be justified in the light of recent work by Mills and Porter (1957) in the United States of America, who showed that driving mileages* above 12,000 miles per year are associated to a significant degree with increased cancer of the lung death frequency among smokers and non-smokers alike of urban survey populations, but not among rural survey populations. However, their conclusion that cigarette smoking acts as the dominant etiological factor in much of today's lung cancer is consistent with the general trend of similar investigations conducted elsewhere. The desirability of supporting this claim by demonstrating the presence of carcinogens in cigarette smoke is obvious. With this in view, the work described in the second part of the present thesis was undertaken. To conclude the Introduction to the thesis, the background to this work is briefly sketched in.

The etiological relationship between tobacco consumption or usage and human cancers arising in sites of exposure, though mooted for many years, did not acquire widespread recognition until the era of large-scale epidemiological studies was initiated at the start of the present decade by Levin, Goldstein and Gerhardt (1950);

* Refers to the number of miles driven by a motorist.

Wynder and Graham (1950) and Doll and Hill (1950). The evidence to date suggests that tobacco represents a carcinogenic hazard (i) in its native state, as witnessed by the high incidence of oral cancers among tobacco chewers, noted by Khanolkar (1944) and by Sanghvi et al. (1955); (ii) in its products of combustion, as witnessed by the lip and mouth cancers associated with pipe and cigar smoking (Levin et al. 1950) and Sadowsky et al. (1953) and lung cancer associated with cigarette smoking. As regards (i), while the possible ancillary roles of chronic irritation and vitamin deficiency, carious teeth and leukoplakia have not yet been evaluated, it would seem that the tobacco leaf itself can contain material which is carcinogenic for the oral mucosa of humans. It is an open question whether the tracheo-bronchial tree is exposed to such material through its back-distillation in the smoking process. However, the tracheo-bronchial tree is exposed to the products of tobacco combustion.

Brief exposure of the tobacco to high temperatures under conditions of oxygen deficiency is encountered in the smoking process - a process which involves a complicated series of changes as yet imperfectly understood (Gilbert and Lindsey 1957). Material formed under similar physical conditions involving thermal decomposition, e.g. coal tar and shale oil are known to contain carcinogens of the aromatic hydrocarbon class. Hence, it seemed reasonable to assume that vegetable matter, after combustion as in

cigarette smoking, should produce representatives of this class of compound, including, possibly, carcinogens. This prediction has been borne out (i) by the bio-assay studies of fractionated cigarette tar carried out by Wynder and Wright (1957) who showed that the carcinogenicity of the "whole" tar resided largely though not exclusively in the neutral fraction; (ii) by the demonstration, spectrophotometrically and spectrographically, of the presence of polycyclic hydrocarbons including the strong carcinogen 3,4-benzopyrene in this fraction.

This carcinogen has been detected in cigarette smoke by many workers (e.g.Cooper and Lindsay (1955), Seelkopt (1955), Lyons (1956)) in concentrations not exceeding 2 parts per million. Wynder and Wright (1957) showed that such a concentration of 3,4-benzopyrene was insufficient to account for the biological activity of their tobacco tar and suggested the possible presence of other carcinogens of the same class. This was not unexpected. Many workers investigating carcinogenic tars and oils (see Discussion) had shown that no parity existed between benzopyrene content and biological activity, and that chromatographic fractions demonstrably free of benzopyrene were carcinogenic, i.e. that other carcinogens were present. The analogy with cigarette tar was strong. Part of the present studies were directed towards the demonstration of carcinogens other than benzopyrene in cigarette smoke. An initial report has been published (Lyons and Johnston 1957b).

If it be decided, as is not unreasonable, that the spectrum of carcinogenic hydrocarbons present in cigarette smoke does not differ significantly from that of soots to which non-smokers are exposed, the unique etiological position of cigarette smoke will have to be supported by the consideration of other factors, one or more of which may operate in conjunction with the carcinogenic hydrocarbons to effect an enhancement of carcinogenic potency. The most important of these factors appeared to be:

(a) that cigarette smoke contains additional carcinogens of a chemical class not met with in atmospheric pollution.

(b) that cigarette smoke, in addition to "formal" carcinogens, contains promoting agents.

(c) that the carcinogens of cigarette smoke exist in a physical state not met in atmospheric soots such as allows their ready access to the cell.

The title of the present thesis required examination of factor (a) above - though the possibility of direct relationship between it and factors (b) and (c) was not overlooked. To this end the following line of investigation was pursued.

Ingram and co-workers have shown that free-radicals are produced when carbonaceous solids are heated up to temperatures of about 550°C when a maximum concentration of radicals is produced (Ingram et al. 1954). Since a similar temperature exists in the burning cigarette, it was considered a possibility that molecules of similar electron imbalance are formed in the smoking process, and

inspired in the main-stream smoke.

Many workers have assigned a role in carcinogenesis to the agency of free-radicals. The action of ionising radiation and radiomimetic agents in this respect has been discussed by Brues and Barron (1951). Oppenheimer et al. (1953) state that many, if not all, carcinogens are compounds capable of forming free-radicals, which may be stabilised as ions. The same workers discussed their possible role in the production of tumours in rodents by implanted films of various high polymers (Oppenheimer et al. 1955). In view of these observations it seemed desirable to investigate cigarette smoke for the presence of free-radicals as a possible parameter in the etiology of lung cancer. A report on their detection and estimation has been published (Lyons, Gibson and Ingram 1958). A more extensive account of the work, which involved electron resonance spectroscopy, is given in the present thesis. An additional approach to the same subject was made using the stable free-radical $\alpha\alpha'$-diphenyl-β-picrylhydrazyl (D.P.P.H.). Using this reagent, light-sensitive components of cigarette, pipe and cigar smoke were detected and studied. Such components were not found in general atmospheric soots.

METHODS.

Procedure adopted in the analysis of particulate phase atmospheric pollutants for the presence of aromatic polycyclic hydrocarbons.

1. Collection of Samples.

(a) Automobile Exhaust Soot. The twin exhaust pipes of a 3.6 litre Ford V8 Pilot car, in good running order, were muzzled with fine hemp sacks and an exhaust sample collected over a two-day period of normal urban motoring.

(b) Diesel Engine Exhaust Soot. A sample was collected in a manner similar to that described in (a) from what was believed to be an efficiently-operating public Leyland double-decker omnibus as the engine idled during the warm-up period. Kotin et al. (1955) have remarked that a diesel engine, in marked contrast to a petrol-consuming engine, can operate under all conditions except warm-up without the attendant production of significant amounts of soot and accompanying hydrocarbons.

(c) A sample of general atmospheric soot was collected over a three-months period during late autumn and early winter in fibre glass filters on a hospital roof in the city of Glasgow.

2. Extraction of Aromatic Hydrocarbons.

10 to 60 gm. quantities of the various soots were extracted in a Soxhlet apparatus with re-distilled fluorescent-free acetone.

In this series of experiments and in experiments with cigarette smoke to be described later, the possibility of contamination by extraneous fluorescent material was excluded by ensuring that reagents and apparatus were free of any trace of fluorescence before the experiments were commenced.

When the extracting acetone appeared fluorescent-free, as judged by inspection under a ultra-violet lamp, reflux was terminated. Following removal of a sample for dry weight determination, the bulk of the extracts, usually dark brown in colour, were taken to dryness and the residues extracted by refluxing for three three-hour periods with petroleum ether (B.P. 60-80°C.). The combined extracts in each case were taken to small volumes of 20-30 ml., in anticipation of fractionation by adsorption chromatography.

3. Adsorption Chromatography of Soot Extracts.

The column liquid chromatogram technique was routinely employed in the present investigations. This technique has been successfully used in the past for the separation and isolation of aromatic polycyclic hydrocarbons from crude mixtures. Thus, Berenblum (1945) isolated 3,4-benzopyrene from coal tar by chromatography on alumina. Berenblum and Schoental (1943) used adsorption chromatography on alumina in conjunction with fluorescence spectrography as the principal method of isolating carcinogenic fractions from blue shale oil and later from horizontal retort tar (1947).

Smith, Sunderland and Sugiura (1951) used fractional elution from silica gel as the means of demonstrating that the carcinogenicity of certain petroleum products resided in the aromatic fraction. Poel and Kammer (1957) and Lijinsky and co-workers (1957) detected members of the aromatic polycyclic hydrocarbon class in creosote oils using adsorption chromatography on alumina. Clemo (1946) isolated carcinogenic aromatic fractions from atmospheric soot and Waller (1952) estimated 3,4-benzopyrene from fractions of urban atmospheres in England. Kotin, Falk and Thomas (1954,5), by a similar method, determined aromatic hydrocarbons from vehicular exhausts, and Kuratsune and Hueper (1958), aromatic hydrocarbons from coffee soots. The method has been used to determine aromatic hydrocarbons from cigarette smoke, by Cooper et al. (1954), Seelkopf (1955), Latarjet et al. (1956), Bonnet and Neukomm (1956), and Wynder and Wright (1957). The diversity of the crude starting materials serves to emphasise the general usefulness of the method.

The methodology in all these experiments involved the adsorption of the material to be separated on a column of adsorbent, usually alumina or silica gel, from a solvent of low eluting power, e.g. petroleum ether, and the elution of separated components in flowing chromatogram by increase in the eluting power of the solvent.

A high degree of empiricism exists in adsorption chromato-

graphy, which is not encountered in other forms of chromatography. This is especially true in the analysis of crude mixtures, when the nature of the mixture, the choice of solvent and adsorbent, the activity of the adsorbent, each constitutes a determinant.

The following procedure was employed in the present investigations.

(a) Preparation of Column: General. Pyrex glass tubes ranging from ½ cm. to 4 cm. in internal diameter were used, the larger of which carried ground-glass joints to which solvent reservoirs could be attached. The lower ends of the tubes were constricted to a stopcock to help regulate the rate of flow. The adsorbent, of a working column height from 6 to 12 times the internal diameter of the tubes, was supported on a plug of solvent-soaked cotton wool resting on the shoulder formed by the constriction above the stopcock. Adsorbent was mixed with solvent in a beaker and poured into the tube carefully and with constant tapping in small portions to ensure regular sedimentation. The solvent was allowed to run through the column whilst packing to diminish eddies and swirls in the liquid. When the level of the adsorbent was at the desired working height, the solvent was allowed to run until its surface was just broken by the protruding particles of adsorbent. A filter paper disc cut to size was placed on top of the adsorbent and the mixture to be separated applied carefully in concentrated solution of solvent by means of a Pasteur pipette. The solvent

was allowed to run until its surface was again broken by the filter paper disc. One or more small quantities of solvent were applied separately in a similar manner to remove traces of the analytical sample from the wall of the tube and carry it down into the column. The test sample was thus contained in as narrow a band as possible just below the top surface of the column. The space above the column was then filled with solvent and the column allowed to run. In the present work it was never found necessary to apply pressure to increase the flow-rate.

(b) Chromatographic Separation (General for soots and cigarette smoke tar). A 3 to 6 gm. quantity of test material in 20-30 ml. solution in petroleum ether was applied to a column of untreated type "I alumina (100-200 mesh, Spence and Co., and originally B.D.H.Ltd.). The column dimensions were approximately 30 x 4 cms. To avoid incidental photo-oxidation of the aromatic hydrocarbons when undergoing separation on the columns, these were shielded from diffuse daylight by wrappings of black paper. The separations were followed by periodic inspection of the columns and eluates under a filtered ultra-violet lamp, which revealed the passage of fluorescent zone(s) on the column. In the initial chromatogram being described 200-400 ml. fractions were normally collected.

The column was initially developed with petroleum ether (60-80°C. B.P.) when a clear blue-fluorescent (or in the case of cigarette smoke tar, a greenish-blue fluorescent) material was observed

to pass quickly through the column. This was collected in the first eluate 0-400 ml. Development was continued with the petroleum-ether until a certain point (vide infra) was reached when the petroleum ether was mixed with progressively increasing quantities of either chloroform or acetone (latterly). Increasing the polarity of the solvent thus was designed to expedite elution of more strongly adsorbed compounds. The point at which it was commenced was decided by observing the behaviour of pure compounds on a control column which was developed in parallel. This column was of the same adsorbent, had similar dimensions and was developed at the same flow-rate (approximately 5 mm. per minute) as the analytical column. 100 µg quantities of 3,4-benzopyrene and fluoranthene were added to this column. When the fluoranthene was about to be eluted, increase in the eluting power of the solvent in the analytical column was commenced. The control column was similarly treated and the chromatographic behaviour of the 3,4-benzopyrene served as a useful guide to that compound's behaviour on the analytical column, cognizance being taken of its possible greater mobility in this column due to displacement effects of other components. Chromatography was terminated when little fluorescent material passed into the receiving flasks and when such material afforded no evidence of fluorescence banding or absorption peaks in the region 240-460 mµ of the spectrum.

Screening by fluorescence spectrography (vide infra) was then performed and as spectral band patterns were rarely discernible against a general area of absorption, each fraction was separately chromatographed on 20 x 2.5 cms. columns of 100-200 mesh alumina. The fractions eluted before the point of chloroform or acetone incorporation in the solvent were chromatographed using petroleum ether as eluent. The remaining fractions were chromatographed using petroleum ether containing 2-8 per cent acetone. Of various solvent mixtures tried, petroleum ether containing low concentrations of acetone were found to be the most suitable for the separation of the crude mixtures of higher aromatic hydrocarbons. The various sub-fractions were purified by chromatography on 100-200 mesh silica gel (Light and Co.) using petroleum ether or petroleum ether containing low proportions of benzene. Alumina and silica gel were employed alternately until compounds had been purified sufficiently for attempts at identification by spectrographic and spectroscopic methods. Silica gel proved useful in retaining yellow and orange coloured impurities on the columns while allowing the polycyclic hydrocarbons to be eluted.

4. Fluorescence Spectrography of Fractions.

Fluorescence spectrography has been established as a rapid means of assessing the successful fractionation of the aromatic hydrocarbons from crude mixtures. In the case of coal tar it proved invaluable in the hands of Cook and co-workers (1933), and

Hieger (1937). Some controversy arose as to the specificity of fluorescence spectra in mixtures of hydrocarbons (Sannie, 1936), but more recent studies (Hieger 1937; Miller and Baumann 1943; Berenblum and Schoental 1943, 1947; Schoental 1957) have vindicated its usefulness.

The characteristic fluorescence spectra of hydrocarbons are retained in the spectrum given by a mixture of hydrocarbons roughly in proportion to the intensity and contrast of the individual bands (Hieger 1937). Spectra may be obtained from minute amounts of material. This is true for all compounds which have a strong fluorescence intensity, e.g. 3,4-benzopyrene. In crude mixtures other compounds occur such as pyrene, chrysene and phenanthrene, which have a comparatively low fluorescence intensity (see Berenblum and Schoental 1946) and which are best identified spectrophotometrically.

For the present studies, a Hilger E.3 Medium Quartz Spectrograph was used. Excitation radiation of 365 mμ was obtained from an Osira mercury vapour lamp with Woods glass filter. The lamp was housed in a box with a window, below which was a condensing lens which focussed the beam on the test sample. This was contained in a small thin glass-walled cell of ½ ml. capacity, held in position immediately in front of the spectrograph aperture. The spectrograph was at an angle of 90° approximately to the beam of incident light. The slit-width was maintained at 0.05 mm. and

the length of the aperture at 4 mm. Spectroscopic cyclohexane was normally employed as solvent. Exposures on Ilford HP3 panchromatic plates varied from 3 to 15 minutes depending on the fluorescence intensity of the sample. For graphic representation of fluorescence spectra, a photoelectric densitometer connected to a Cambridge recorder was employed. Fluorescence maxima were calculated from the 3650 A mercury line by use of a standard curve derived from the Hartmann dispersion formula.

5. Ultra-Violet Absorption Spectrophotometry.

A Uvispek spectrophotometer was used which had been calibrated against a mercury spectrum. Spectroscopic cyclohexane and benzene or occasionally 95 per cent ethanol were used as solvents. Samples, in 1 cm. cells, were scanned normally in the spectrum range 460 mμ to 260 mμ at 2 mμ intervals. When peaks became apparent, intervals in the peak region were cut to 0.5 mμ. Where compounds were present in not too low a concentration, dilution was adjusted such that absorption occurred in the accurate instrumental region of 0.1 to 0.8.

To plot the spectrum of an unknown compound the following procedure was adopted. The molecular weight was taken to be 200 and the concentration in moles per litre, C. These values are substituted in the equation -

$$\text{Log } e = \text{Log } \frac{A.M}{b.C}$$

where e , A, M, C, and b have the usual significance.

It follows that -

$$\text{Log } e = \text{Log } A + 2.3010 - \text{Log } C$$

or $\text{Log } e + X = \text{Log } A$, when $X = \text{Log } C - 2.3010$.

The Log of the absorbance is plotted against wavelength.

The estimation of polycyclic aromatic hydrocarbons. The method described by Cooper (1954) was employed, which utilises the base-line technique. The method assumes Beer's law and linear background absorption over short distances. Determinations were carried out using a fixed suitable slit-width and at an optical density not greater than 0.7. Definitive peaks at the higher wavelengths of the spectra were chosen as these were normally associated with less background absorption.

A line was drawn between two points at the base of a peak, to complete the greatest triangle. The height of the peak was measured from a point on the base-line vertically below it. The same procedure was adopted with a standard solution of the hydrocarbon adjusted to approximately the same absorption level as the analytical. The approximate concentration of the analytical solution was calculated by simple proportion.

The following peaks and slit-widths were used in the determinations:

	Peak	Slit-width
Anthracene,	376 mμ	0.14 mm.
Pyrene,	334 mμ	0.24 mm.
Fluoranthene,	288 mμ	0.44 mm.
1,2-Benzanthracene,	385 mμ	0.12 mm.
1,2-Benzopyrene,	332 mμ	0.24 mm.
3,4-Benzopyrene,	385 mμ	0.12 mm.
1,12-Benzperylene,	387 mμ	0.12 mm.
3,4,8,9-Dibenzopyrene,	314 mμ	0.28 mm.
1,2,3,4-Dibenzopyrene,	332 mμ	0.24 mm.
11,12-Benzfluoranthene	380 mμ	0.12 mμ.

6. Investigations of Cigarette Smoke.

Procedure employed in the investigation of cigarette smoke for the presence of aromatic polycyclic hydrocarbons.

(a) Type of Cigarette used. Cigarettes of a type commonly smoked in the United Kingdom were used. These were provided by The Imperial Tobacco Company and were obtained through the Medical Research Council. Equal numbers of four unnamed brands, A, B, C and D, were used. The average weight of a cigarette was 1.1 gr. with a moisture content of about 11 per cent.

(b) Cigarette Smoking and the Collection of Smoke Products. The cigarettes were smoked in a manner designed to simulate the human method of smoking. As the human method varies from individual to individual, there did not seem to be any valid reason for employing a strictly regulated averaged method of smoking as is used by some other workers in this field

Therefore for the present investigation it was decided to vary the tempo or rate of smoking within limits which were thought to coincide more with reality.

Two- to four-second draws were made during the course of smoking, two draws on average being made per minute. The time taken to smoke a cigarette to a butt length of about 1.5 cms. was varied between seven and twelve minutes.

The smoking unit consisted of a manifold (Plate 1) designed to hold a charge of 24 cigarettes vertically in positions equidistant from a pressure outlet so that an even rate of burning was achieved. The pressure outlet was connected to a train which consisted of the following component parts in series: (a) 2-litre round-bottom flasks each charged with about 0.75 litre re-distilled acetone. The inlet tubes were of 1 cm. diameter and led down to a distance 0.5 cm. from the bottoms of the flasks. The charged flasks were cooled to a temperature of about -70°C by an acetone-CO_2 mixture prior to smoking and kept at this temperature throughout smoking by immersion in vacuum flasks containing the refrigerant. (b) Two Dreschel bottles of 250 ml. capacity containing re-distilled acetone at room temperature. On smoking a batch of 500 cigarettes, most of the products appeared in the round-bottom flasks. The terminal Dreschel bottle was observed to contain little fluorescent material, signifying efficient trapping of smoke tar. (c) The terminal Dreschel bottle was connected to a reservoir to which was attached a lead to a boosted filter-pump which was in continuous operation during smoking; a mercury manometer so that pressure drops due to

blockage in the train or temporary failure in the filter-pump could be detected; an air-leak consisting of a short length of rubber tubing connected to a stopcock which was either operated manually - closed to effect a draw on the cigarettes - or operated mechanically (when required) by placing the shaft of the stopcock between the jaws of a gear which was driven by a small electric motor, so as to make one rotation (to give two draws) per minute.

(c) Preparation of Neutral Aromatic Fraction from Trapped Smoke Products. The acetone smoke solutions were combined and a 20 ml. sample withdrawn for dry weight estimation. The acetone was distilled off at reduced pressure leaving a brown tar which was solid at room temperature and mobile at a temperature of about 50°C. As the acetone distillate was slightly yellow in colour, this was re-distilled leaving a further minute quantity of tar, which was added to the bulk.

The tar was next refluxed for three 3-hour periods with charges of fluorescent-free petroleum ether (60-80°C. B.P.). The combined petroleum ether extracts were taken to a convenient volume (about 200 ml.) and a 5 ml. portion withdrawn for dry weight estimation. The bulk, when cool, was extracted, successively, with three portions each of 2N.HCl to remove basic components, and 2N.NaOH, to remove acidic components. The solution was finally washed twice with distilled water, after which it was

dried by shaking with a quantity of anhydrous Na_2SO_4. A 5 ml. portion was removed for dry weight estimation. The remainder was concentrated to a volume of about 30 ml. for chromatographic separation.

A check was made on possible losses in the recovery of aromatic hydrocarbons in the above procedure. The following experiment was performed.

To an acetone solution of smoke products from 50 cigarettes, 50 µg of 3,4-benzopyrene in acetone was added. The chemical manipulation was exactly as described above. On chromatography of the petroleum ether extract on 100-200 mesh alumina using petroleum ether as solvent initially, followed by a mixture of petroleum ether and chloroform up to a ratio of 9 to 1 parts, an estimated 74 per cent. of the benzopyrene was recovered. This result can be compared more than favourably with that of Wynder and Wright (1957) who showed that up to 40 per cent of the aromatic hydrocarbons may become soluble and taken over into the aqueous phase during extraction with dilute acid.

(d) The Chromatographic and Scanning Techniques were similar to those applied in the case of the soots.

7. The Distribution of an Aromatic Polycyclic Hydrocarbon on Smoking.

The estimation of 3,4-benzopyrene, added to cigarettes prior to smoking, in main-stream and side-stream smoke, butts and ash.

(Note: The smoke which is drawn through the cigarette - and inspired by the smoker - is termed "main-stream" smoke while that which issues from the smouldering coal or tip into the general atmosphere is called "side-stream" smoke).

Fifty cigarettes were immersed end on, in an ethereal solution containing 500 µg of 3,4-benzopyrene. The solution was allowed to soak up two-thirds of the length of the cigarettes. The ether was allowed to evaporate at room temperature. The untreated ends of the cigarettes were placed in the manifold and the cigarettes were smoked as described in 6(b). The side-stream was collected by clamping an inverted glass funnel over the burning cigarettes in the manifold and drawing the smoke, which issued from the burning coals (tips) through a re-distilled acetone trap (Plate 2). When smoking was completed, the funnel and lead tube were washed free of condensed smoke with acetone. To this solution was added the products collected in the acetone trap.

The butts, of length about 1.5 cms., and ash, were each shaken with quantities of petroleum ether. The solid phase was removed in both cases by filtration. Neutral aromatic fractions of the main-stream and side-stream smoke and butt extracts were prepared as described in 6(c) and chromatographed on short columns of alumina. A petroleum ether extract of the ash was applied straight on to a prepared chromatography column.

8. Control Experiment on Possible Atmospheric Soot Contamination of Cigarette Smoke Products.

A control experiment was performed to determine whether the soot contamination of the air drawn through the train during smoking could significantly affect the analysis of aromatic hydrocarbons in the smoke products collected during a typical operation - the smoking of a batch of 500 cigarettes.

The average volume of air used per draw was found to be 35 ml. If 20 draws were made per cigarette smoked, the quantity of air consumed per 500 cigarettes is 20 x 35 x 500 = 350 L of air.

Approximately this quantity of air as measured by a flowmeter was drawn through two Dreschel bottles containing acetone. A slight blue fluorescence was produced in the first trap. The acetone was removed by distillation and the residue extracted with hot petroleum ether. This extract, on chromatography on a small column (8 x 1 cms.) of alumina yielded a trace of what was probably pyrene as judged by a slight absorption maximum at 334 mμ. Pyrene was found to occur in greatest concentration in atmospheric soot (see under Results). Therefore contamination of cigarette smoke products from this source was considered negligible.

9. Temperature Measurements during Cigarette Smoking.

Temperature measurements were made using an iron-constantan

thermocouple, constructed to specifications and calibrated by Dr. M. Bluhm of the Regional Physics Department, Glasgow. The thermocouple hot junction measured 2 mm. in length and the conducting elements were insulated by a heat-resisting material (shellac). The cold junction was kept at 0°C. in an ice-water trough. mV readings were made on a sensitive galvanometer and temperatures read from a standard curve. For measurements, the thermocouple was both inserted laterally and threaded lengthwise. As temperatures were expected to vary with intensity of suction, readings were taken in the burning coal without suction, with the suction employed during 2- to 4-second draws. The readings were compared with those obtained during average human smoking. The thermocouple was inserted at various distances from the burning coal and an estimate of the temperature gradient obtained. Averages of twenty readings were taken in each case.

10. Measurement of Free Radical Content of Cigarette Smoke Products. Collection of Sample of Cigarette Smoke Condensate at Liquid Oxygen Temperatures.

The vessel shown in Plate 3 was employed. This consisted of a manifold chamber which was constructed to accommodate four cigarettes in the upright position. An outlet tube laterally placed led to an air reservoir and filter pump, as in the normal smoking apparatus described above. A sealed off length of ½"

diameter thin-walled glass tubing led down from the centre of the floor of the manifold chamber. This tube, which was about 4" in length, was placed in liquid O_2 during smoking. The design of the apparatus was such that it could be accommodated in the resonance spectrometer for direct measurements. A glass rod, surrounded by a baffle within the chamber, and held so that its end was about ½ cm. from the opening of the tube, was incorporated into the apparatus and served as a plunger to free the tube from blockage. A coil of thick gauge copper wire was wound round the exterior of the lower part of the chamber from which two leads were led into the refrigerant. This had the effect of keeping the lower portion of the manifold chamber cool (by conduction) and facilitating the condensation of the smoke, which was found to condense in the tube as a pale yellow solid. This turned brown on rise of temperature.

When about ½ gm. of condensate had been collected, the apparatus was quickly transferred to the electron resonance spectrometer and the tube was inserted into the centre of a H_{014} cavity, surrounded by liquid oxygen. The free-radical concentration of the deep-frozen material was then determined using 100 KC/s modulation and phase-sensitive detection so that the free-radical absorption was traced out as a derivative on the pen recorder.

The smoking apparatus was then removed from the spectrometer

and the condensate in the tube was warmed to 60°C. for about 5 minutes so that any active radicals could re-combine. The tube and contents were again deep-frozen, re-inserted in the spectrometer and the radical concentration again measured. In this way it was possible to distinguish between short-lived active radicals and radicals that were stabilised by resonance or stabilised sterically (Ingram and Tapley 1955).

For the purpose of ascertaining whether free-radicals were formed in the cigarette smoking process and, this being the case, to carry out some investigations into their nature and stability, the Paramagnetic Resonance Absorption technique was employed. This method is most specific for the detection of free-radicals, which can be estimated against a large diamagnetic background or in the presence of other paramagnetic material such as ions of the transition group of elements. Details of the theory and circuits involved can be obtained in standard works such as "Spectroscopy at Microwave Frequency" (Ingram 1955).

The method detects the absorption of microwave energy which results from the interaction between a variable external magnetic field and the magnetic moments of unpaired electrons present in the sample.

In the present instance, an X-band spectrometer was used which measured the absorption of 9,000-megacycle (3.2 cms.) microwave energy incident upon the sample, as influenced by the

variation of the strength of an externally applied magnetic field. The magnetic moment of an unpaired electron leads to absorption of 9,000-megacycle/s radiation at an external magnetic field of 3,300 oersteds.

Incident microwave power was generated by a Klystron. It was transmitted via a wave-guide to a H_{014} resonance cavity containing an opening to accommodate the sample tube. The output from the cavity was detected by a silicon diode (crystal). The external magnetic field was swept slowly through the range in which absorption was expected, and in addition, was varied or modulated sinusoidally over a smaller range of a fraction of the width of the absorption peak, (see diagram). The frequency of this modulation was 100 kilocycles/sec. The signal from the crystal was amplified and fed into a phase sensitive detector which selects the signal (which is coherent with the modulation of the magnetic field) and rejects most of the background noise (which is incoherent). The signal is recorded as the sweeping of the magnetic field proceeds and the resulting record on the pen recorder is a derivative of absorption with respect to magnetic field.

11. Experiments involving the use of Stable Free Radical αα'-Diphenyl-β-Picryl Hydrazyl (D.P.P.H.).

References regarding the synthesis and properties of D.P.P.H. are as follows: Goldschmidt and Renn (1922); Poirier, Kahler and

Bennington (1953); Braude, Brook and Linstead (1954); Blois (1955).

D.P.P.H. is a stable free radical giving no evidence of chain initiator properties. It does not combine with atmospheric oxygen and hence may find use in combustion processes which involve oxygen - such as the process of cigarette smoking. It is a violet-coloured solid readily soluble in benzene. Because of its odd electron D.P.P.H. shows a strong absorption band at 520 mμ in benzene. Reactions in benzene solution can be followed by observing colour depletion, i.e. decrease in absorbance, at this wavelength, the decrease in absorbance being stoichiometric with respect to the number of electrons taken up.

The chemical reactivity of free radicals is due to the available combining energy of the odd electron(s), and their reactions, whenever possible result in the completion of electron pairs. Thus, D.P.P.H. has found use as a free radical scavenger. It also reacts, however, with compounds having reversible oxidisable groups, e.g. sulphydryl groups, hydroquinone systems (Blois 1958) according to the mechanism:-

$$\text{(a)}\quad (\text{D.P.P.H.})\cdot + \text{R} - \text{SH} \rightarrow (\text{D.P.P.H.}){:}\text{H} + \text{R} - \text{S}\cdot$$

$$2\ \text{R} - \text{S}\cdot \rightarrow \text{R} - \text{S} - \text{S} - \text{R}$$

$$\text{(b)}\quad (\text{D.P.P.H.})\cdot + \text{HO} - \text{R} - \text{OH} \rightarrow (\text{D.P.P.H.}){:}\text{H} + \text{HO} - \text{R} - \text{O}\cdot$$

$$(\text{D.P.P.H.})\cdot + \text{HO} - \text{R} - \text{O}\cdot \rightarrow (\text{D.P.P.H.}){:}\text{H} + \text{O} = \text{R} = \text{O}$$

Therefore D.P.P.H. can be used to measure anti-oxidant activity (Blois 1958).

In the present series of experiments efforts were made to distinguish between the free radical scavenging activity and the oxidant activity of D.P.P.H.

It has been found that D.P.P.H. reacts with cigarette, pipe and cigar smoke, extracts of unsmoked cigarettes, and extracts of atmospheric soot. For a series of hydroquinone standards ranging from 2.00 to 0.66 10^{-4} M per L, it was found that the absorption readings, taken 1 minute after addition of D.P.P.H., was proportional to the concentration of the standards. The adsorption values lay between 0.18 and 0.60. In the experiments to be described, the absorption readings at 1 minute were normally taken as a measure of the D.P.P.H. activity of a solution.

A working concentration of 10^{-4} M D.P.P.H. was used throughout the experiments. This has an absorption value of 0.916. A stock solution of molarity 10^{-2} was stored in a ground-glass stoppered flask which was shielded from diffuse daylight. The relationship between absorption and concentration over the accurate range of absorption values (0.1 to 0.8) was linear, i.e. Beer's law was applicable over this range. 10 ml. quantities of test material in duplicate were used throughout, to which 0.1 ml. quantities of stock solution D.P.P.H. (10^{-2} M) were added from a fast-delivery pipette.

It was found that certain test samples were light-sensitive. In efforts to quantitate this finding, test solutions (in benzene) were irradiated for varying periods of time. 10 ml. quantities were delivered by a burette into 15 ml. capacity ground-glass stoppered tubes which were placed in a rack at a distance of 25 cms. from an open-arc mercury lamp. The solutions were kept cool during irradiation by a fan. At 60-minute intervals from the start of exposure, pairs of tubes were withdrawn and 0.1 ml. stock solution of D.P.P.H. added to each tube. The colour depletion was followed on the spectrophotometer.

12. Fluorescence Measurements on Cigarette Smoke.

The H730 fluorescence attachment in conjunction with the Uvispek spectrophotometer was used. The excitation radiation was 365 mμ. Cells of approximately 18 ml. capacity provided with black glass lids were used. When filling cells care was taken to prevent the formation of air bubbles in the liquid and to avoid leaving a meniscus under the black lid.

For benzene solutions of cigarette smoke a fluorescence concentration equivalent to about 80 per cent. of a 0.13 mg. per cent. quinone sulphate in 0.1 NH_2SO_4 standard was used, as this value lay on the descending portion of the fluorescence-concentration curve (see Bowen and Wokes 1953), i.e. where fluorescence intensity was linear with concentration.

PLATE 1. Smoking manifold with part of train.

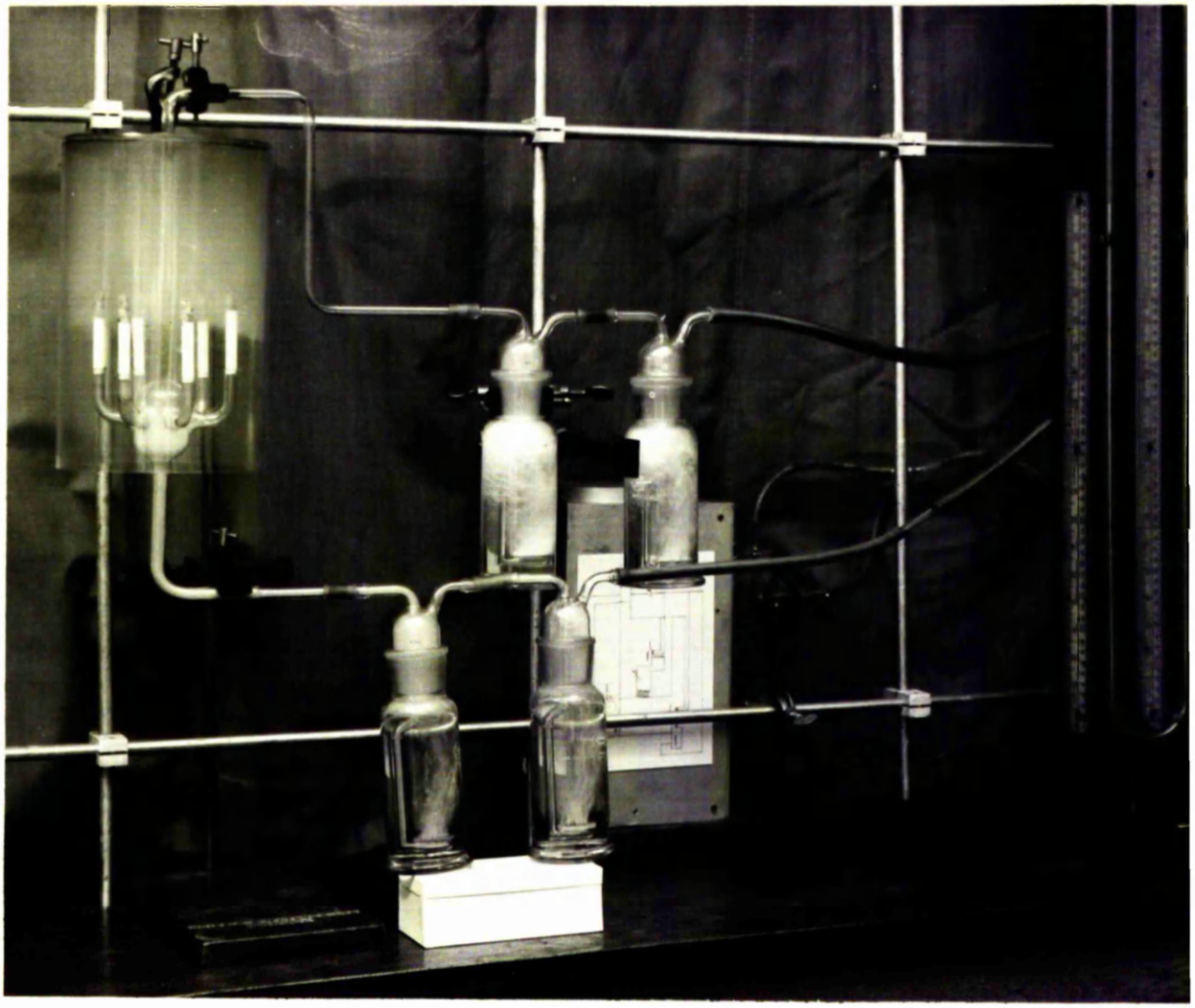


PLATE 2. Arrangement for trapping side-stream smoke.

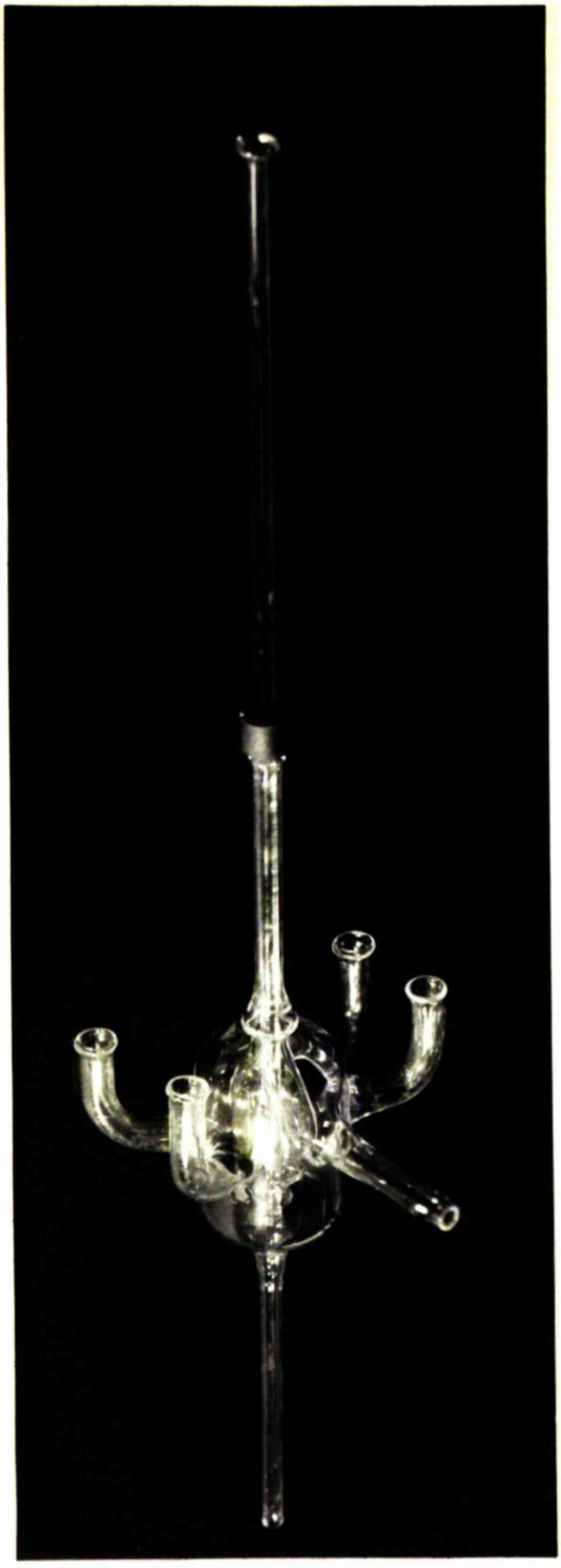


PLATE 3. Smoking apparatus as used with the Electron Resonance Spectrometer.

R E S U L T S

PART ONE. PARTICULATE AIR POLLUTANTS

General plan of presentation

The results obtained from the analysis of the three soots, General Atmospheric Soot (A), Diesel Exhaust Soot (B) and Petrol Exhaust Soot (C), are presented in turn. A final section (D) is devoted to a comparison of the findings made in the preceding sections.

In sections (A), (B) and (C), tables, giving an indication of the profile of the initial chromatograms before the commencement of gradient elution, are given. Further tables follow giving the absorption and fluorescence features of the various chromatographic fractions obtained and the compounds suggested by them. Plates, on which the fluorescence spectra obtained are reproduced are next included, followed by figures giving the absorption spectra of compounds which have been detected. The absorption spectra which are presented are those of carcinogens and of compounds not previously detected in

the soots under investigation. The nomenclature adopted throughout the thesis is that of Clar (1952) except in one instance, where Tetraphene is called by its more familiar name, 1,2-Benzanthracene.

Section (A). Atmospheric Soot.

54.001 g. of dry soot was used in the present analysis. It yielded 3.012 g. of Petroleum-Ether extract, representing a 5.6% yield. This extract, which had a dark brown colour, was subjected to chromatographic separation as described in "Methods". The profile of the initial chromotogram on development with 2.5 l. Petroleum-Ether, i.e. at a stage immediately before the incorporation of chloroform in the solvent is shown in Table 1.

Six main fractions, A - F, were eluted. Apart from fraction A, which was colourless, all fractions had a yellow to orange colour. Following careful removal of the solvent under pressure, the oily residues were taken up in 10 - 15 ml of Petroleum-Ether and rechromatographed on 20 x 2.4 cms columns of Alumina.

Fraction A yielded a fast moving dull violet fluorescent material in the initial eluate. Rechromatography

on a long column of Alumina (30 x 1 cms) yielded fractions with absorption maxima in the region of 320 mμ, 287 mμ, 276 mμ, 268 mμ, suggesting the presence of Naphthalene and Methyl analogues. The fluorescence spectra consisted of uncharacteristic three-banded systems in the region 375 - 435 mμ.

A diffuse violet fluorescent zone was next eluted having the absorption peaks of Acenaphthalene. Anthracene was detected in the succeeding eluates of this fraction by its absorption maxima at 374, 354 and 251 mμ.

Fraction B was chromatographed using Petroleum-Ether alone as solvent. An initial violet fluorescent eluate presented peaks at 293, 281 and 274 mμ suggestive of Phenanthrene. A light blue and a violet fluorescent zone were subsequently eluted which showed the presence of Pyrene and an Anthracene derivative respectively by their absorption characteristics.

For Fraction C, 0 - 5% Chloroform was incorporated in the solvent. Four main fractions were separated which presented the absorption characteristics of Fluoranthene, Chrysene and 1,2-Benzanthracene and a violet fluorescent

component which presented absorption maxima at 380, 360, 338 and 288 mμ and fluorescence bands at 380, 405 and 430 mμ.

For fraction D, 0 - 10 % Chloroform was incorporated in the solvent. 3,4- Benzopyrene was detected in this fraction by its absorption and fluorescence spectra. It was preceded by fractions which contained Perylene and 1,2 - Benzopyrene, (suggested by their fluorescence and absorption characteristics), and succeeded by a fraction which had absorption maxima at 387, 367 and 302 mμ, and which were indicative of 1,12 - Benzoperylene.

Fraction E was eluted with solvent containing 15% Chloroform. The following sub-fractions were obtained: (i) a blue-violet fluorescent component showing a fluorescent band at 370 mμ with a further Anthracene or Phenanthrene-like system with bands commencing at 388, 408 and 435 mμ. (Plate 4, Fr. E1). On rechromatography a fraction was isolated which had the absorption characteristics of 3,4 - Benzofluoranthene (Clar, 1952: p 405). The absorption spectrum obtained is presented (Fig.2 (b)).
(ii) a blue fluorescent component having fluorescence maxima at 413 and 437 mμ and absorption peaks at 412, 384, 362, 287 and 272 mμ. (iii) traces of a light blue

fluorescent compound with fluorescence bands starting at 434 and 464 mu.

Fraction F was chromatographed using chloroform up to a concentration of 20% in the solvent. Four main components were obtained: (i) a violet fluorescent material which gave fluorescence bands at 393, 415 and 442 mu, and absorption maxima at 393, 382, 365, 335, 328, 320, 313, 303, 287 (max.) and 270 mu. Trace quantities of a fraction with main fluorescence bands at 397 and 418 mu. This resembles the fluorescence spectrum of 1,2,6,7 - Dobenzopyrene. Absorption data were however inconclusive and the trace quantity of material available did not allow of further purification. (iii) blue violet component identified as 11,12-Benzofluoranthene by its fluorescence and absorption spectra. (iv) blue fluorescent component with fluorescence maxima at 403, 417, 430 mu and absorption maxima at 402, 369, 300 and 282 mu.

Fraction G yielded three main components: (i) a dull violet material which had a fluorescence band at 352 mu. (ii) a violet material giving a fluted fluorescence spectrum with bands commencing at 380, 400 and 425 mu. Absorption maxima were obtained at 380, 360, 352, 337, 320 309, 299, 272, 266 mu. The absorption spectrum is presented (Fig. 2a). (iii) In this fraction fluorescence bands were

visible against considerable background at 378 and 398 mμ. Apart from giving inflexion points at 378 and 352 mμ no absorption peak was obtained until a maximum at 262 mμ.

Fraction II, in which a blue fluorescent material was eluted clear of the pink zone mentioned in the Profile (Table 1) yielded four components: (i) a blue-violet fluorescent component which had a fluted fluorescence spectrum with bands at 396, 420 and 445 mμ and an absorption maximum at 395 mμ. (ii) blue fluorescence, with fluorescence maxima at 410, 435 and 465 mμ, and absorption maxima at 410, 388, 360, 322, 298 and 254 mμ. (iii) a light blue fluorescent component with fluorescence bands at 441 and 470 mμ and absorption maxima at 438, 414, 300 and 290 mμ. (iv) a blue-violet fluorescent material showing fluorescence bands at 377, 397 and 420 mμ, and absorption maxima at 396, 376, 364, 312, 300, 290 mμ.

The above findings are listed in Table 2. In Plate 4 the fluorescence spectra given by compounds are presented. The absorption spectrum of the 3,4-Benzopyrene, the carcinogen which was found to occur in highest concentration is presented (Fig. 1b). The spectrum of the 1,2 Benzanthracene

is also shown (Fig. 1a). The Pyrene and 3,4-Benzo-pyrene, Anthracene and 1,2-Benzanthracene were estimated after repeated alternate chromatography on Alumina and Silica Gel. The concentrations are given in Section D.

Table 1

Profile of initial chromatogram on development with about 2.5 l. Petroleum-Ether - at the stage immediately before the incorparation of Chloroform in the solvent.

Main Fraction	Zone Distance from top of column in cms.	Observation
H	0 - 4.0	Dark brown colour. Non-fluorescent pink colour.
H	4·0 - 5·0	Dull reddish fluorescence.
G	5·0 - 5·5	Yellow-orange colour. Bright yellow white fl.
F	5·5 - 6.6	Yellow band. Blue fluorescence.
E	6·6 - 8·0	Light yellow zone. L-Blue fluorescence.
D	9·0 - 15·0	Light yellow zone. Blue fluorescence.
C	17·5 - 21·0	Light yellow zone. Blue violet fluorescence.
B	21·5 - 26·0	Light yellow zone. Blue fluorescence.
B	26·5 - 29·0	Colourless. Light blue fluorescence.

Table 2

Compounds detected in a sample of General Atmospheric Soot.

A : Absorption maxima (mμ)
F: Fluorescence maxima (mμ)

Fraction		Spectroscopic Features	Compound
A1	A	320, 287, 276, 268 mμ	Naphthalene and simple derivatives
	F	-	
A2	A	340, 324 mμ	Acenaphthalene
	F	-	
A3	A	376, 355, 251	Anthracene
	F	378, 395, 420	
B1	A	293, 281, 274	Phenanthrene
	F	-	
B2	A	372, 362, 356, 335, 318, 305, 272.	Pyrene
	F	-	
B3	A	380, 359, 342, 326, 257	Anthracene derivative
	F	380	
C1	A	359, 342, 288, 277	Fluoranthene.
	F	-	
C2	A	385, 359, 344, 290, 280	1,2-Benzanthracene.
	F	385, 404, 428	
C3	A	361, 344, 320, 267, 258.	Chrysene
	F	-	
C4	A	382, 361, 430, 288	
	F	382, 407, 431	
D1	A	366, 332, 317, 290	1,2-Benzopyrene
	F	-	

Table 2 (ctd.)

Fraction	Spectroscopic features	Compound
D2	A 437,411,386 F 437	Perylene (trace)
D3	A 404,385,364,347,297,284. F 404,408,427,454.	3,4-Benzo-pyrene.
D4	A 408,387,368,303,291. F -	1,12-Benzo-perylene.
E1A	A 368,350,302,275,255 F 428,450	3,4-Benzo-fluoranthene.
E1B	A - F 388,410,435	
E2	A 413,384,362,287,272. F 414,437.	
E3	A - F 435,464.	
F1	A 393,382,365,335,328,320, 313,303,287. F 393,415,442.	
F2	A - F 397,418.	
F3	A 401,380,360,309,296. F 401,410,428,456.	11,12-Benzo-fluoranthene.
F4	A 402,369,300,282. F 403,417,430.	
G1	A - F 352.	
G2	A 380,360,352,337,320, 309,299,272,266. F 380,387,399,421	
G3	A 378,352,365 F 378,398.	

Table 2 (ctd.)

Fraction		Spectroscopic features	Compound
H1	A	397	
	F	397, 422, 445	
H2	A	412, 388, 360, 318, 298.	
	F	412, 435, 465.	
H3	A	438, 414, 300, 290.	
	F	441, 470	
H4	A	396, 376, 364, 312, 300, 290.	
	F	377, 397, 420.	

The absorption spectra of fractions A1 to E1 were taken using cyclohexane as solvent. The remainder were taken using Benzene as solvent.

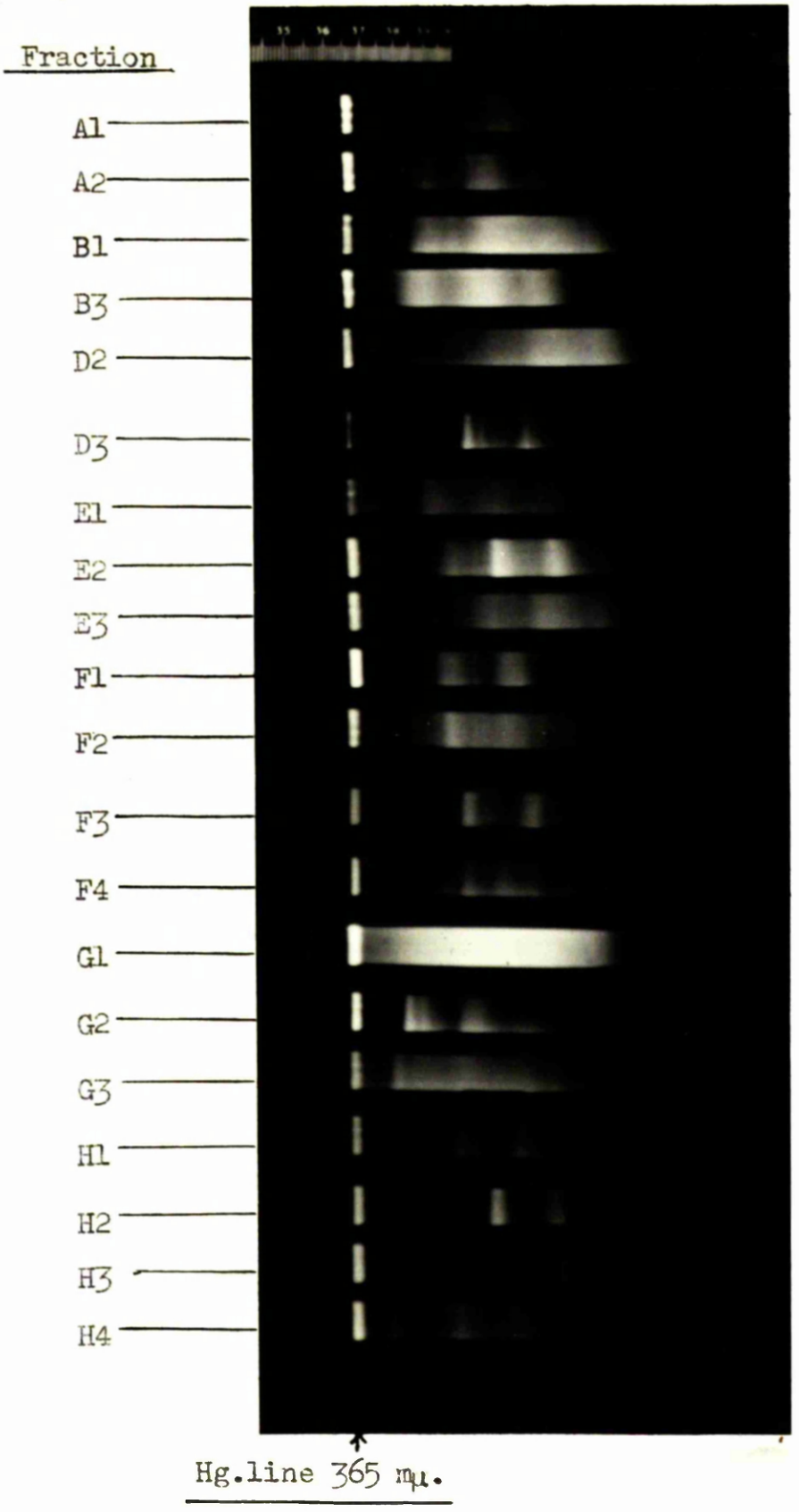
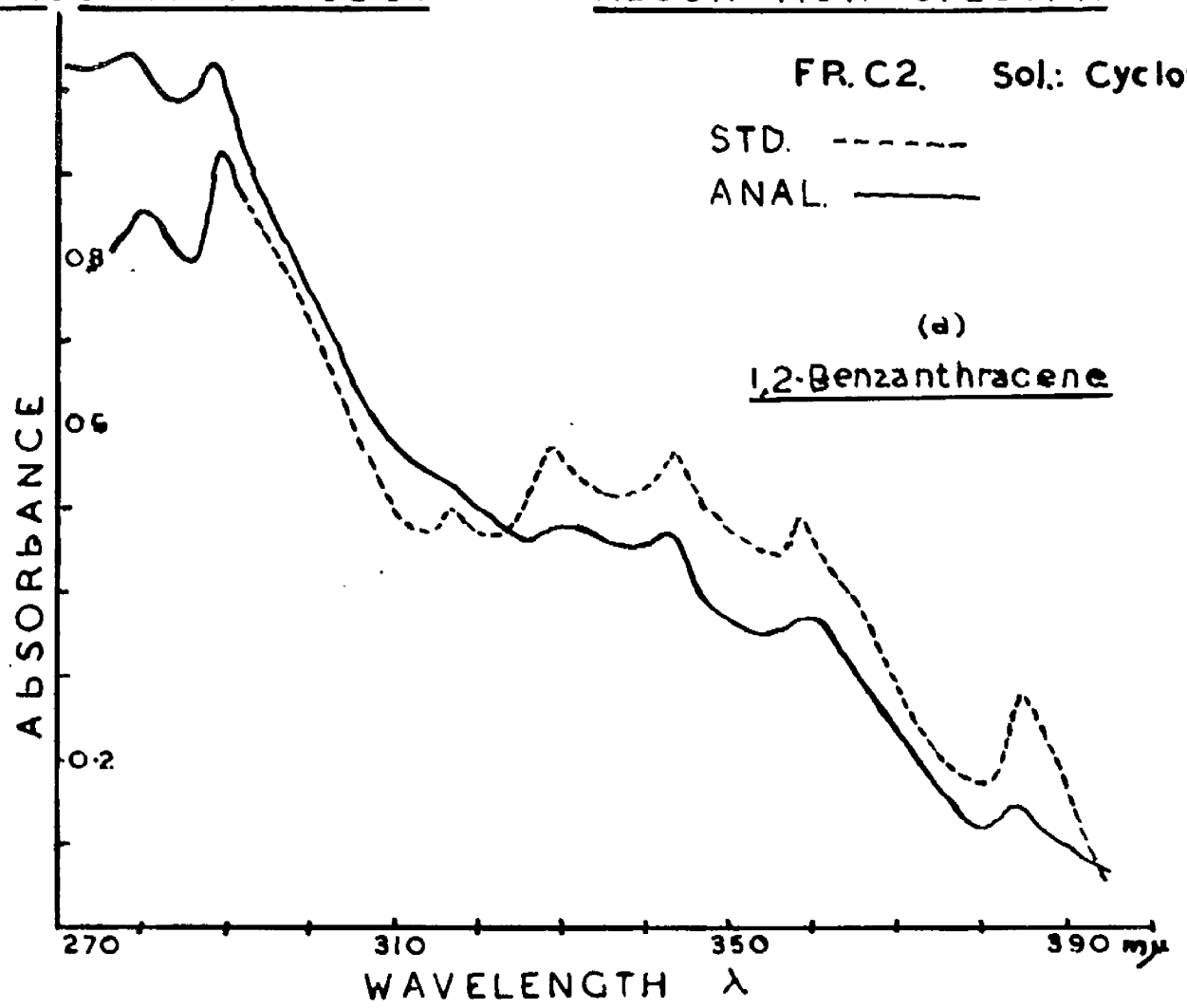


PLATE 4. Fluorescence Spectra of fractions obtained from general atmospheric soot. Solvent: cyclohexane.

ATMOSPHERIC SOOT — ABSORPTION SPECTRA



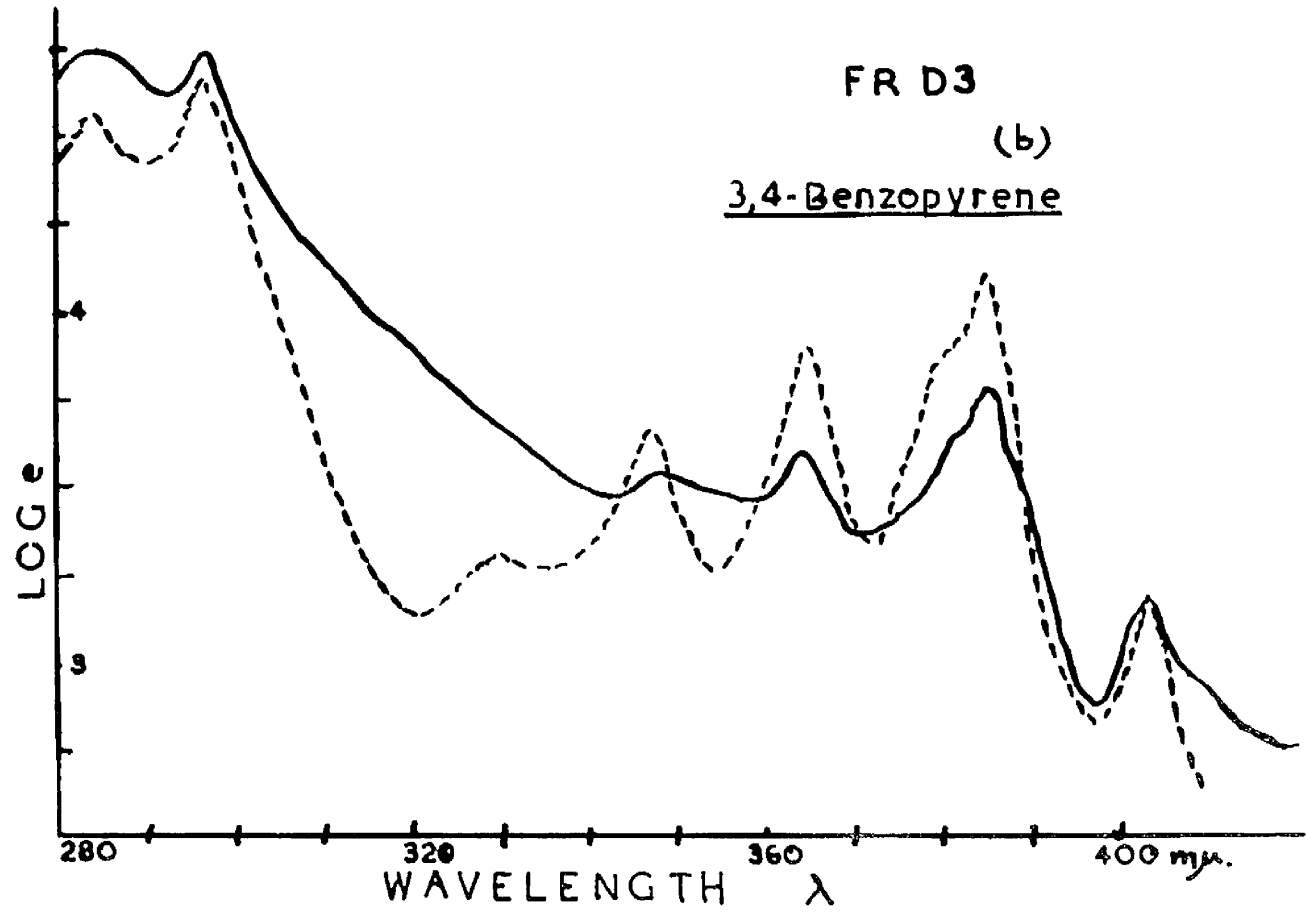
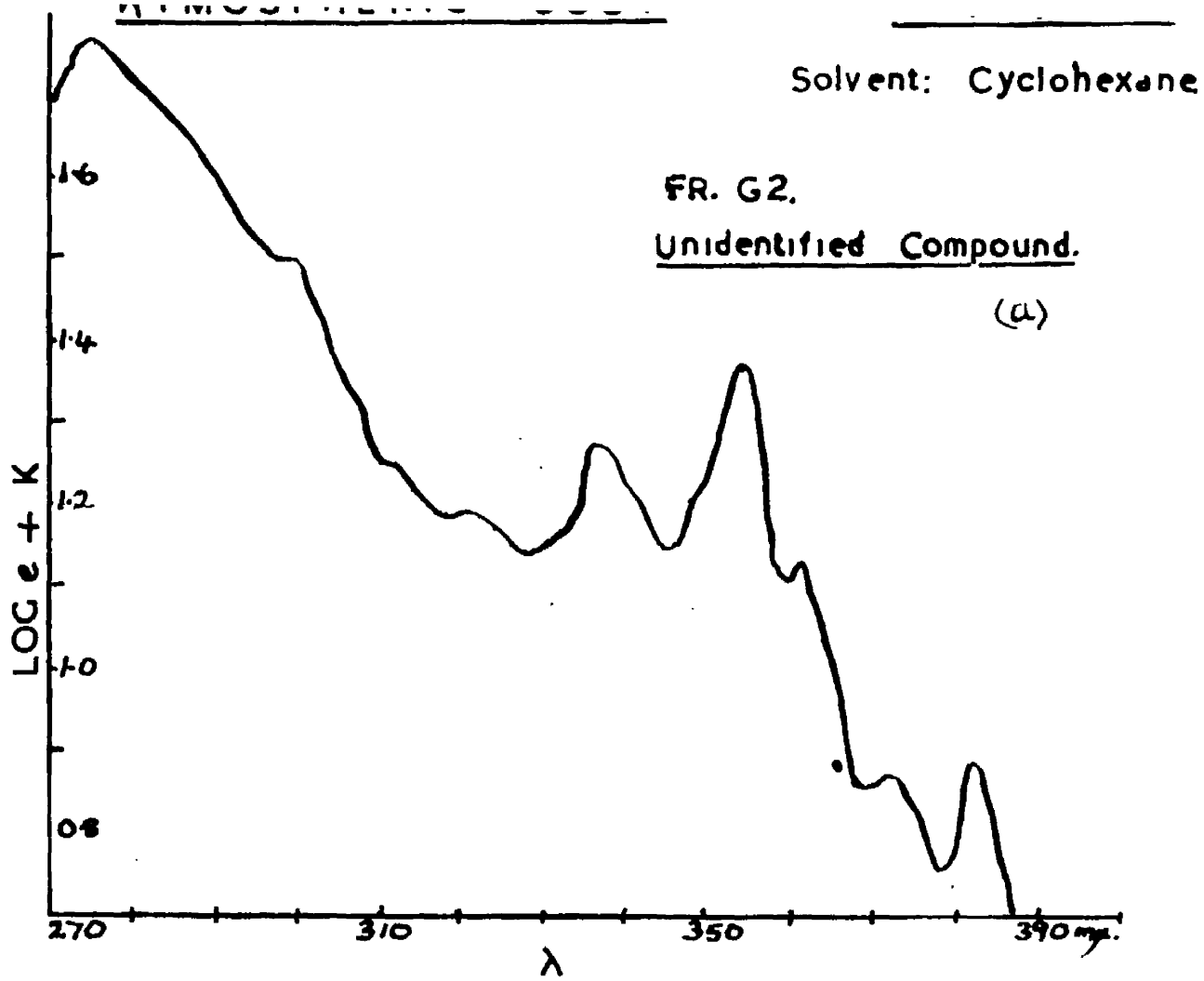


FIG. 1. Solvent: Cyclohexane.


Solvent: Cyclohexane
FR. G2.
Unidentified Compound.
(a)
LOG e + K
1·6
1·4
1·2
1·0
0·8
270
310
350
390 mμ.
λ

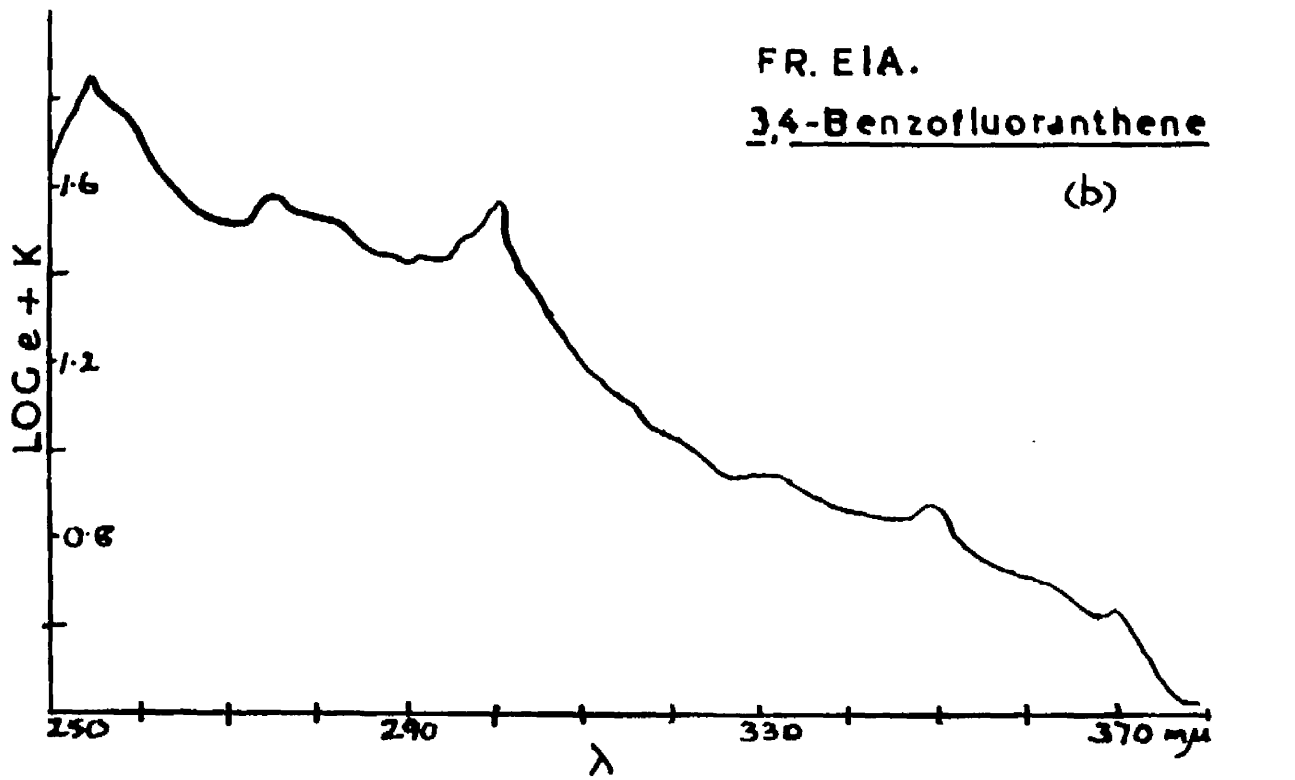

FR. E1A.
3,4-Benzofluoranthene
(b)
LOG e + K
1·6
1·2
0·8
250
290
330
370 mμ
λ

FIG. 2.

Section (B). Diesel Exhaust Soot

12·65 of diesel exhaust soot afforded 4·67 (or 36·9%) of Acetone Extract, 4·60 g of which was soluble in Petroleum-Ether. This solution had a deep orange colour and a pungent odour.

Chromatography was carried out on this extract as previously described. Table 3 shows the chromatogram profile on development with 2·5 l of Petroleum-Ether at a stage before gradient elution was commenced.

A faintly violet-fluorescent material was found to be readily washed through the column (Fraction A). On removal of the solvent a blue-fluorescent oil remained. Part of the fluorescence was removed by repeated filtration of the oil through columns of Alumina and Silica Gel. The oil weighed 2·2 g. Its refractive index was taken using a Abbé Refract-ometer at 20°C. The value obtained was 1·4605. This does not correspond with the values for Methyl or Hydro Naphthalene derivatives. The Refractive Index of a sample of overhead lubricant was 1·4931. The Refractive Index of a sample of diesel fuel was 1·4680. All Refractive Index values were recorded at 20°C. The oil obtained in the present case was considered to be a mixture of lubricant oil and fuel.

It proved a good solvent for crystalline 3,4-Benzopyrene.

The U.V. absorption spectra of blue-violet fluorescent material separated from the oil exhibited peaks at 312, 286 and 275 mu., indicating the presence of Naphthalene or simple derivatives.

Five main fractions were eluted, B F, which were each separately rechromatographed on smaller columns of Alumina as in the case of the atmospheric soot. Following regrouping on the basis of fluorescence and absorption spectral screening, components were further purified by alternate chromatography on Alumina and Silica Gel.

At the termination of elution in the original chromatogram, a brick-red and a salmon coloured zone, each about 0·5 cm in width, were visible 6 and 9 cms from the top of the column. They were non-fluorescent.

The main compounds obtained from the diesel soot are shown in Table 4.

An eluate preceding the 3,4-Benzopyrene showed most of the spectral characteristics of "Compound X" (Kotin & al, 1954). It was associated with an orange colour. No definite peaks were recorded in the visible region of the spectrum however.

The fluorescent spectrum (not shown) showed a series of very faint lines not unlike those observed for Pyrene. The lines of 'Compound X" were not however superimposable on those of Pyrene.

In the initial eluates succeeding the 3,4-Benzopyrene a brilliant blue fluorescent compound was found, (Fr.D6), which is tentatively identified as 3,4-Benzofluoranthene. Pentaphene was detected in Fraction E1. Its absorption spectrum is given (Fig. 3b). This compound has been detected in Cigarette Smoke (Wynder & Wright, 1957) and in Creosote Oils (Lijinsky, Saffioti & Shubik, 1957).

Difficulty was experienced in separating individual components from the succeeding eluates of this fraction. However the blue-fluorescent compound Coronene was identified which was closely followed by a blue-green fluorescent material with a main fluorescence band at 458 mμ. The absorption spectrum, which showed Coronene peaks at 343 and 304 mμ, was not definite enough for characterisation. Following this material a green-fluorescent eluate was obtained which had a fluorescence spectrum corresponding to 1,2,3,4-Dibenzopyrene. The absorption spectrum showed the characteristics of this compound. This, as far as is known, is the first time that this potent carcinogen has been demonstrated in any soot. The same compound was later identified in Petrol Exhaust and cigarette smoke,(see succeeding sections).

Comparatively large quantities of 11,12-Benzofluoranthene were detected by both fluorescence and absorption analysis. This was followed by a light blue component which had the spectral characteristics of the non-carcinogenic 1,2,9,10-Dibenzotetracene (Fig.4b). A violet fluorescent compound was next eluted which had a sharp fluted fluorescence spectrum (F4). This compound also occurred in atmospheric soot (G2). Its absorption spectrum (Fig.2a) was not sufficiently suggestive of any known compound. Fraction F5 in the diesel soot analysis showed a banded fluorescence spectrum and absorption maxima which could not be characterised. The same compound was found in an atmospheric soot fraction (H2).

Table 3

Profile of Initial Chromatogram of Diesel Soot Extract on development with about 2.5 l Petroleum-Ether

Main Fraction	Zone Distance from top of column in cms	Observation
F	0 - 2	Dark brown colour. Non fluorescent.
	3 - 4	Yellow zone. Bright yellow fluorescence.
E	5 - 6	Yellow band. Bright blue fluorescence.
D	6 - 7	Colourless. Blue-Violet fluorescence.
	7.5 - 9.5	Light yellow colour. Blue fluorescence.
C	10.0 - 14.0	Light yellow colour. Light-blue fluorescence.
	15 - 20	Light yellow colour. Blue fluorescence.
B	20 - 24	Light yellow colour. Blue-violet fluorescence.

Table 4

Compounds detected in Diesel Exhaust Soot

A: Absorption maxima (mμ)
F: Fluorescence maxima (mμ)

Fraction		Spectroscopic Features	Compound
B1	A F	376,355, 251 378,395,420	Anthracene
B2	A F	293,281,274 -	Phenanthrene
C1	A F	372,362,356,335,318, 305,272. -	Pyrene
C2	A F	380,359,342,326,257 380	Anthracene derivat- ive. (ive.
C3	A F	359,342,288,277 -	Fluoranthene
C4	A F	376,368,355,338,310, 291,278. -	Orange coloured compound.
C5	A F	385,359,344,290,280 385,404,424.	1,2-Benzanthracene.
D1	A F	366,332,317,290 -	1,2-Benzopyrene
D2	A F	437,411,386 437 - 470.	Perylene
D3	A F	(405),387,360,342,320, 306,292. 385,405,427.	
D4	A F	404,386,365,347,298,284. 404,426,454	3,4-Benzopyrene

Table 4 (ctd.)

Fraction		Spectroscopic Features	Compound
D5	A	407,388,368,303,291	1,12-Benzo-perylene.
	F	-	
D6	A	368,351,303,294,275,256.	3,4-Benzo-fluoranthene.
	f	(395) 428,450.	
D7	A	413,400,383,362,345,317,303.	
	F	413,437.	
E1	A	424,411,399,377,360,349, 317,306.	Pentaphene
	F	423.	
E2	A	428,410,388,342,305,293.	Coronene
	F	-	
E3	A	458,424,414,385,366,341, 327,303,275.	
	F	458,(466),492,526,570.	
E4	A	454,433,403,381,363, 332,317,303.	1,2,3,4-Dib-enzopyrene.
	F	462.	
F1	A	-	
	F	407,332.	
F2	A	401,380,360,309,296.	11,12-Benzo-fluoranthene.
	F	401,410,428,456.	
F3	A	433,407,326,313,300,289.	1,2,9,10-Dib-enzotetracene.
	F	434	
F4	A	380,360,352,337,320,299, 272,264.	
	F	380, (387),399,421.	
F5	A	412,388,359,318,289. 412,435,465.	

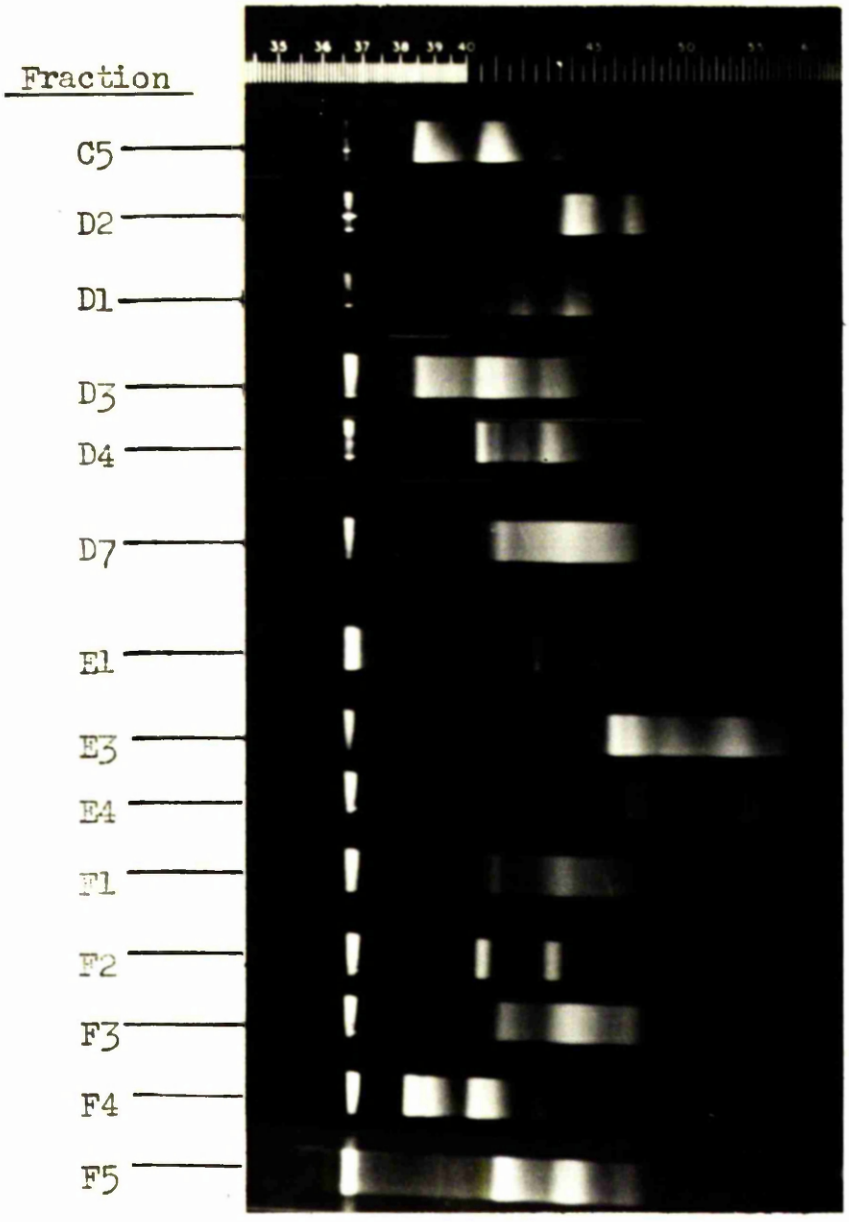
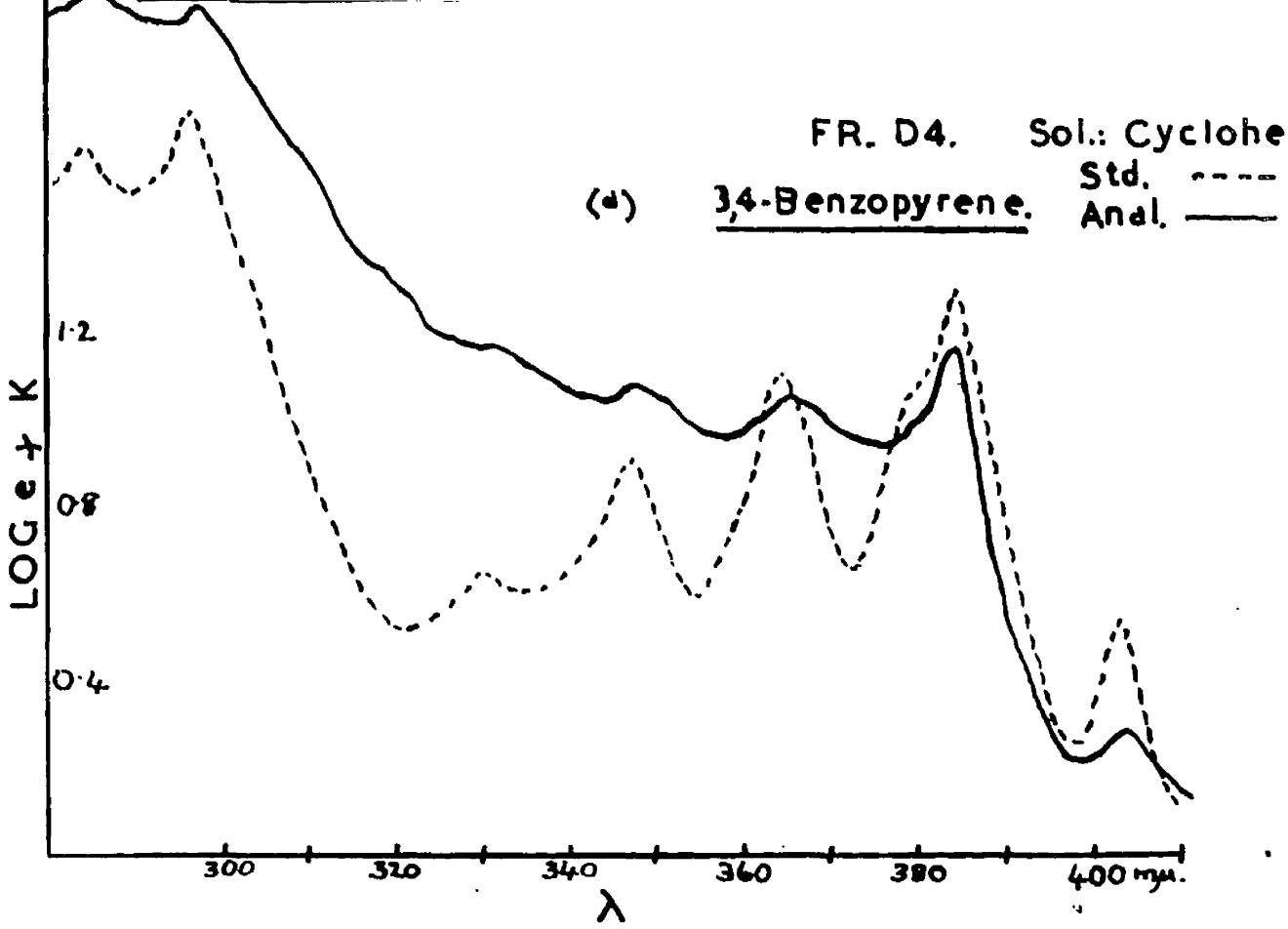


PLATE 5. Fluorescence Spectra of fractions obtained from diesel exhaust soot. Solvent: cyclohexane.


FR. D4. Sol.: Cyclohexa
Std. ----
Anal. ——
(d) 3,4-Benzopyrene.
LOG e + K
1·2
0·8
0·4
300
320
340
360
380
400 mμ.
λ

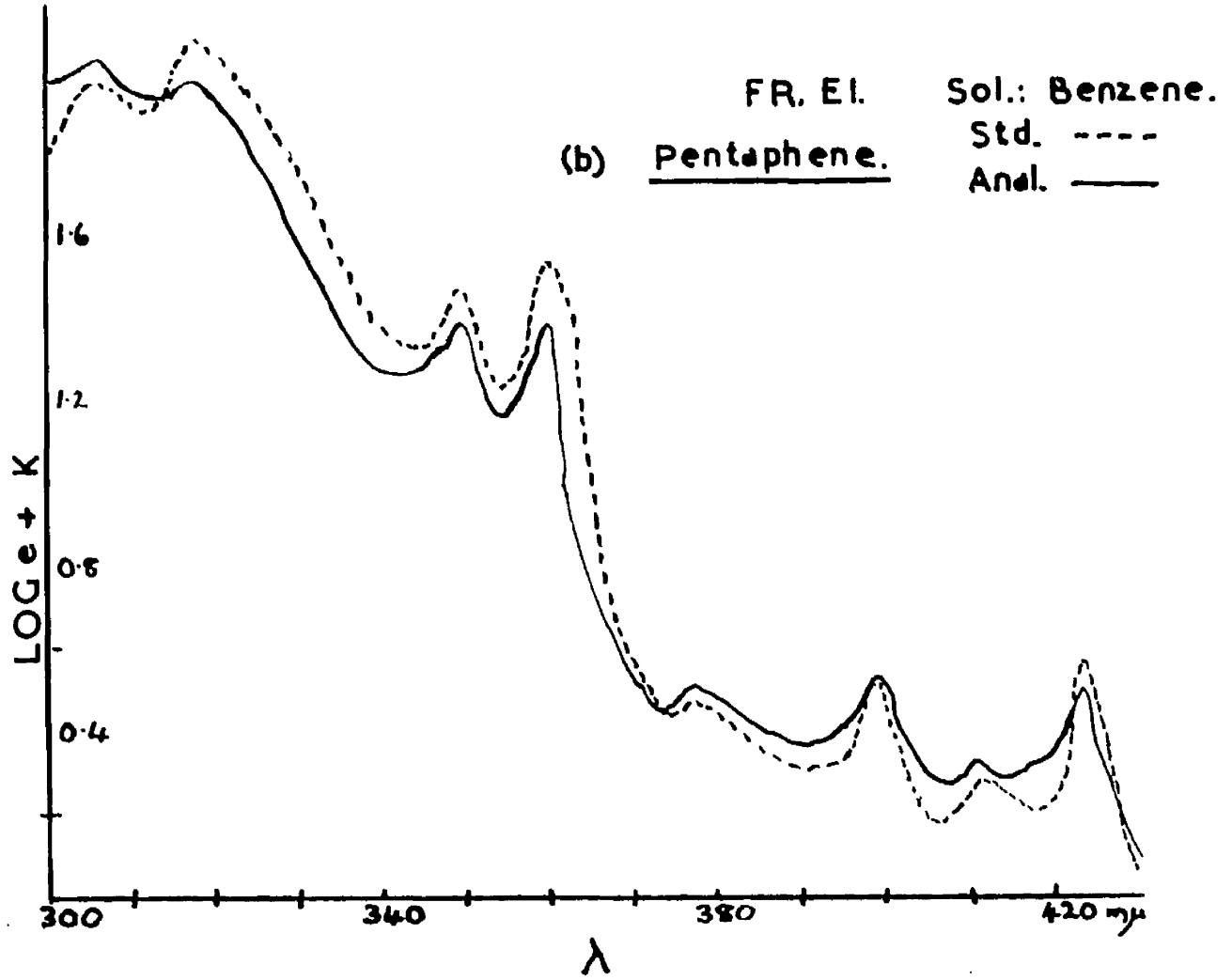
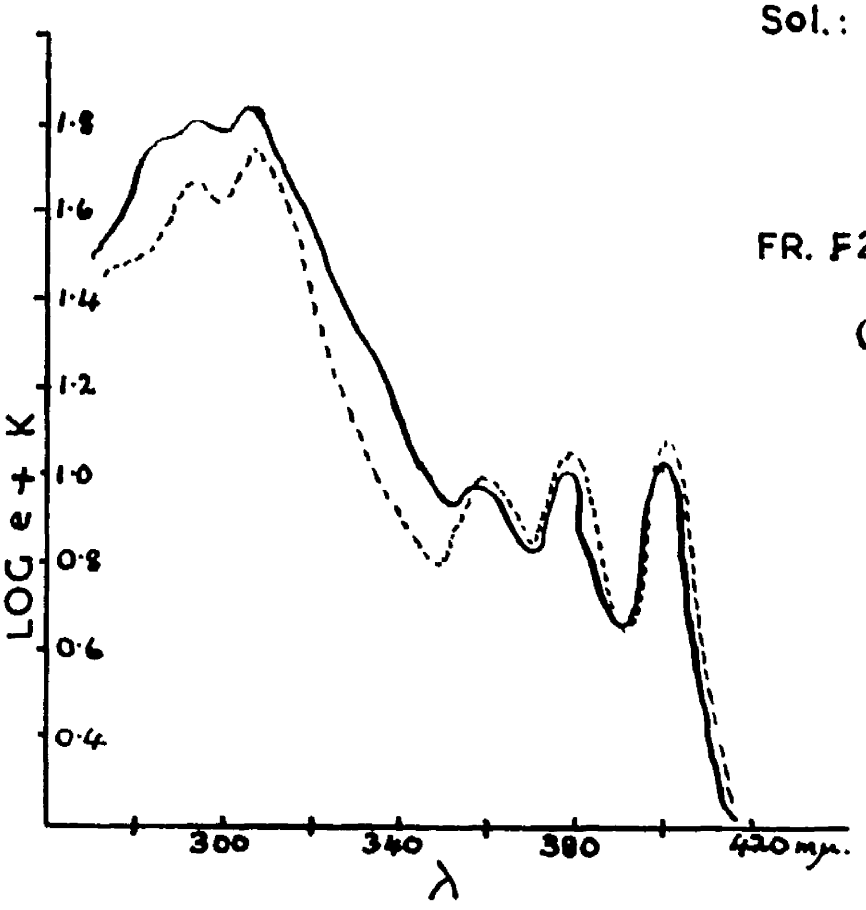

FR. E1. Sol.: Benzene.
Std. ----
Anal. ——
(b) Pentaphene.
LOG e + K
1·6
1·2
0·8
0·4
300
340
380
420 mμ
λ

FIG. 3

DIESEL SOOT

Abs. Spectra

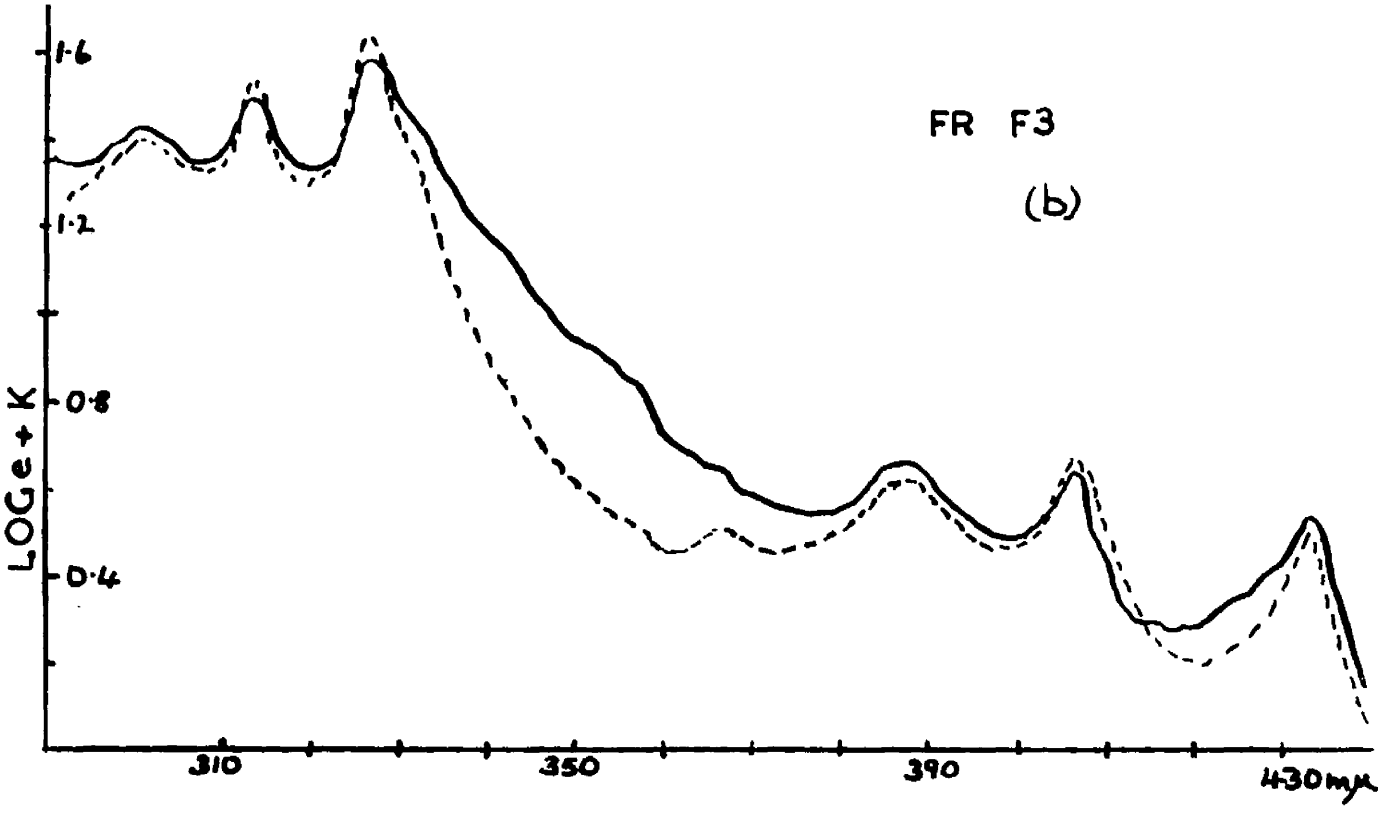
Sol.: Benzene.



FR. F2.

(a)

11, 12-Benzofluoranthene



FR F3

(b)

1,2,9,10-Dibenzotetracene.

FIG. 4

Section (C). Petrol Exhaust Soot

5.87 g of petrol exhaust soot yielded 4.56 (or 77.7%) of Acetone Extract. This extract, which had a yellow colour was completely soluble in Petroleum-Ether.

Chromatography was carried out on the Petroleum-Ether soluble material as described previously. The profile of the initial chromatogram on development with 2.5 l of Petroleum-Ether is shown in Table 5.

As in the case of the Diesel Exhaust Soot a small quantity of colourless oil with a blue fluorescence was recovered in an initial eluate. The oil weighed 1.19 g and had a refractive index of 1.45. Naphthalene and simple derivatives were separated from it on a column of Silica using Petroleum-Ether as eluent.

Eleven succeeding main fractions yielded 55 fractions on chromatography, which were then regrouped on the basis of absorption and fluorescence characteristics into 30 sub-fractions. These were further purified by alternate chromatography on Alumina and Silica Gel, using, in the case of, the more strongly absorbed components on Alumina, Petroleum-Ether containing small percentages of Acetone as

eluent. For the same components on Silica Gel, Peroxide-free Ether was incorporated in the Petroleum-Ether up to a concentration of 10%. The main components detected are listed in Table 6.

The petrol exhaust soot showed a departure from the atmospheric and diesel soots in possessing a number of different components all having the anthracene atom,(i.e. fractions B2,B3,B4,D1,D2), and also a larger quantity of 1,2-Benzanthracene.

In an eluate (Fr.G3) succeeding that containing 1,12-Benzoperylene, a compound which is tentatively identified as 3,4-Benzofluoranthene was detected by its absorption spectrum (Fig.6).

The presence of Tetracene (Naphthracene) was shown in this soot, as well as the Dibenzotetracene previously detected in the Diesel Soot (F3). The unidentified compound with green-blue fluorescence previously demonstrated in the diesel soot was again detected in petrol soot, as were small quantities of the potent carcinogen 1,2,3,4-Dibenzopyrene (Fraction J.2)

Fraction K3 had the absorption and fluorescence characteristics of another strongly carcinogenic dibenzo-

pyrene, 1,2,4,5-Dibenzopyrene. This compound, it is believed, has hitherto not been demonstrated in any pyrogenic material. A reference sample of the pure compound (obtained from Dr. Clar), facilitated the identification. The fluorescence and absorption spectra are presented in Plate 6 and 9 and Fig. 8a respectively. The first fluorescence band corresponds with the 416 mμ absorption peak which is given by Cook, Schoental & Scott (1950) as being the longest U.V. absorption band. The details of the fluorescence and absorption spectra correspond with those of the reference standard. The compound was present in minute concentration (see Table 6).

Less confidence was felt in attempts to identify the next eluate in which a very faint 450 mμ fluorescence band was superimposed on another system having maxima at 422 and 445 mμ (Fraction K4). Some absorption maxima characteristic of the carcinogen 3,4,8,9-Dibenzopyrene were obtained (Fig. 7a).

Fractions K5 and K6 were obtained on further chromatography from a blue-fluorescent eluate which had a striking fluorescence spectrum (XVII Lyons & Johnston, 1957). In that paper the absorption spectra of both K5 and K6 were presented i.e Figs. 4 and 5 respectively. K5 on purification afforded

an absorption spectrum (see Fig. 8b), which seemed identical with that of 1,12,2,3-Dibenzoperylene (see Clar p.302). The first fluorescence maximum at 405 mμ corresponded with the 405 mμ absorption maximum of the Dibenzoperylene. The absorption peak noted by Clar (loc.cit) at 421·5 mμ is of low intensity and, as with the 428 mμ peak in 1,2,4,5-Dibenzopyrene (Clar, loc.cit) and the 420 and 428 mμ peaks in Coronene, may not appear in the fluorescence spectrum. It was noted that the first fluorescence band obtained by Brocklehurst (1953) for Coronene was at 410 mμ and not at the expected longer maximum 428 mμ. The latter fluorescence data relating to Coronene was verified in the present instance. A reference standard of 1,12,2,3-Dibenzoperylene was not available during the present studies. K6 was not identified, nor was L1. The absorption spectrum of K6 is presented (Fig. 9a). L2 afforded fluorescence and absorption evidence suggestive of 3,4-Benzotetraphene (Fig. 9b). Doubt is cast upon this evidence, from observations on the chromatographic sequence, as more complex molecules seem to have been eluted in preceding eluates. It is stressed however that,as overlapping in the main fractions, J,K,L etc., occurred, the order of elution as presented in the table and plate is not necessarily correct,e.g.K5 or K6 may have slower chromatographic mobility than L1 or L2. This

holds for the other analyses in the present series of investigations. Fractions L3 and L4 were not identified. Fractions L2 and L3 which had main fluorescence bands at 391 and 385 mμ in Cyclohexane resemble spectrographically fractions isolated from horizontal retort tar by Berenblum and Schoental (1947) who showed such fractions to be carcinogenic for rabbit skin. In the present instance, however, with a main fluorescence band at 391 mμ, fraction L3 gave an absorption spectrum suggestive of 3,4-Benzotetraphene which is non-carcinogenic.

Table 5

Profile of Initial Chromatogram of Petrol Soot Extract on development with about 2.5 l Petroleum-Ether

Main Fraction	Zone Distance from top of column in cms	Observation
L	0 - 2	Dark brown colour. Non-fluorescent.
K	2 - 2.5	Yellow orange band. Blue-white fluorescence.
J	2.5 - 4.0	Yellow colour. Yellow fluorescence.
H	4.5 - 5.5	Yellow colour. Light-blue fluorescence.
G	7.0 - 10.0	Yellow orange colour. Blue-violet fluorescence.
F	10.5 - 12.0	Colourless. Light-blue fluorescence.
E	12.0 - 17.0	Colourless. Violet fluorescence.
D	19.0 - 22.0	Light yellow colour. Blue-violet fluorescence.
C	24.0 - 26.0	Light yellow colour. Blue fluorescence.
B	27.0 - 29.0	Light yellow colour. Violet fluorescence.

Table 6

Compounds detected in Petrol Exhaust Soot

A: Absorption maxima (mμ)
B: Fluorescence maxima (mμ)

Fraction		Spectroscopic Features	Compound
B1	A F	376,355,228, 324,251 378,395,420	Anthracene
B2	A F	386,365,347,260 387,407.	Anthracene derivative.
B3	A F	380,359,342,326,257 382,403,428.	Anthracene derivative.
B4	A F	389,368,349,258. 390,412,440.	Anthracene derivative.
C1	A F	372, 362,335,319,305. -	Pyrene.
C2	A F	359,342,288,277. -	Fluoranthene.
C3	A F	377,342,328,276,264. -	3-Methylpyrene(?)
C4	A F	376,368,355,338, 324, 310,291,278. -	Orange compound.
D1	A F	388,362,346,264. 389,410,435.	Anthracene derivative.
D2	A F	386,360,348,336, 322, 292, 274,257. 386,403,428.	Anthracene derivative?

Table 6 (ctd.)

Fraction		Spectroscopic Features	Compound
E1	A	385, 359, 344, 290, 280	1,2-Benzanthracene.
	F	385, 404, 424.	
F1	A	388, 366, 332, 317, 304, 290, 278.	1,2-Benzopyrene.
	F	-	
F2	A	437, 411, 386	Perylene.
	F	437, 464.	
G1	A	404, 386, 365, 347, 298, 284.	3,4-Benzopyrene.
	F	404, (407), 426, 454.	
G2	A	407, 388, 368, 303, 291.	1,12-Benzoperylene.
	F	-	
G3	A	370, 350, 302, 294, 276.	3,4-Benzofluoranthene.
	F	(395) 428, 450.	
G4	A	432, 408, 390.	Anthanthrene.
	F	429, 458.	
H1	A	471, 441, 415, 393, 274.	Tetracene.
	F	471	
H2	A	423, 411, 400, 360, 349, 317, 306.	Pentaphene.
	F	-	
H3	A	428, 410, 388, 342, 305, 293.	Coronene.
	F	-	
J1	A	458, 424, 414, 384, 366, 342, 303.	
	F	458, (466), 492, 526, 570.	
J2	A	454, 433, 403, 381, 332, 317.	1,2,3,4-Dibenzopyrene.
	F	462.	

Table 6 (ctd.)

Fraction		Spectroscopic Features	Compound
J3	A	401, 380, 360, 309, 296	11,12-Benzofluoranthene.
	F	401, (410), 427, 456.	
J4	A	433, 407, 326, 313, 300, 289.	1,2,9,10-Dibenzotetracene.
	F	434.	
K1	A	414, 405, 381, 328, 312, 304.	
	F	414, 437.	
K2	A	-	
	F	410, 435.	
K3	A	428, 416, 395, 378, 360, 327, 306, 296.	1,2,4,5-Dibenzopyrene.
	F	416, (426, 430), 440.	
K4	A	422, 412.	
	F	422, 445.	
	A	452, 424, 402, 314, 300.	3,4,8,9-Dibenzopyrene.
	F	450 (faint).	
K5	A	422, 405, 391, 377, 357, 344, 309, 297.	
	F	404, 418, 428, 443, 453.	1,12,2,3-Dibenzoperylene?
K6	A	(422, 405), 396, 369, 347, 328, 304 (296).	
	F	450, 467, 478.	
L1	A	405, 391, 380, 340, 319, 309, 294.	
	F	405, 430.	

Table 6 (ctd.)

Fraction		Spectroscopic Features	Compound
L2	A	391,383,366,348,331,306, 290.(Cyclohexane).	3,4-Benzotetraphene?
	F	391,414,340.	
L3	A	385,363, 349, 336,312, 302.	
	F	385,407,334.	
L4	A	400,390,378, 356,352, 327,312,298,290.	
	F	400,425,455.	

Note: Absorption spectra of fractions B1 to G4 refer to Cyclohexane as solvent, while the remainder refer to Benzene (unless specifically stated).

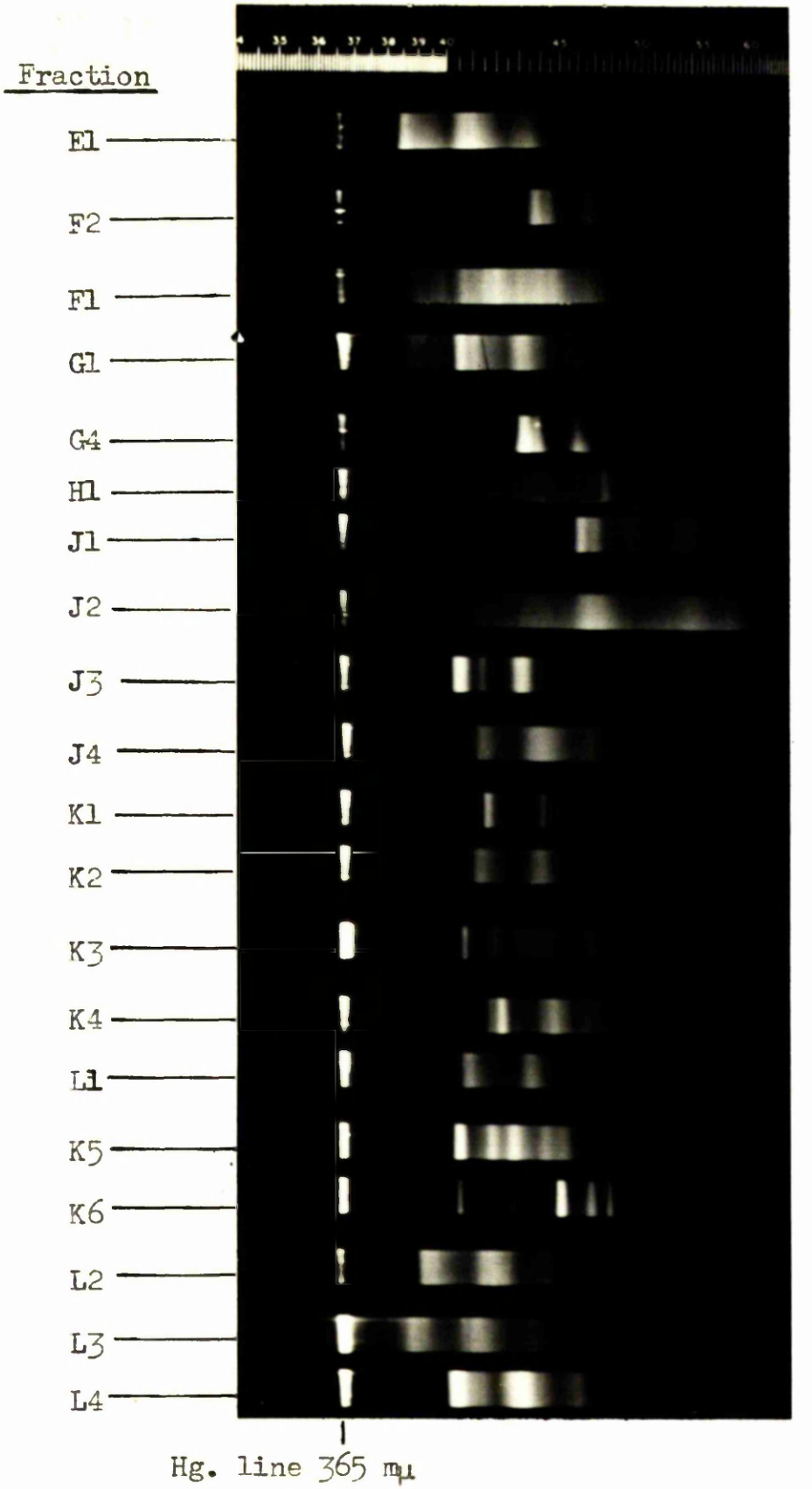


PLATE 6. Fluorescence Spectra of fractions obtained from petrol exhaust soot. Solvent: cyclohexane.

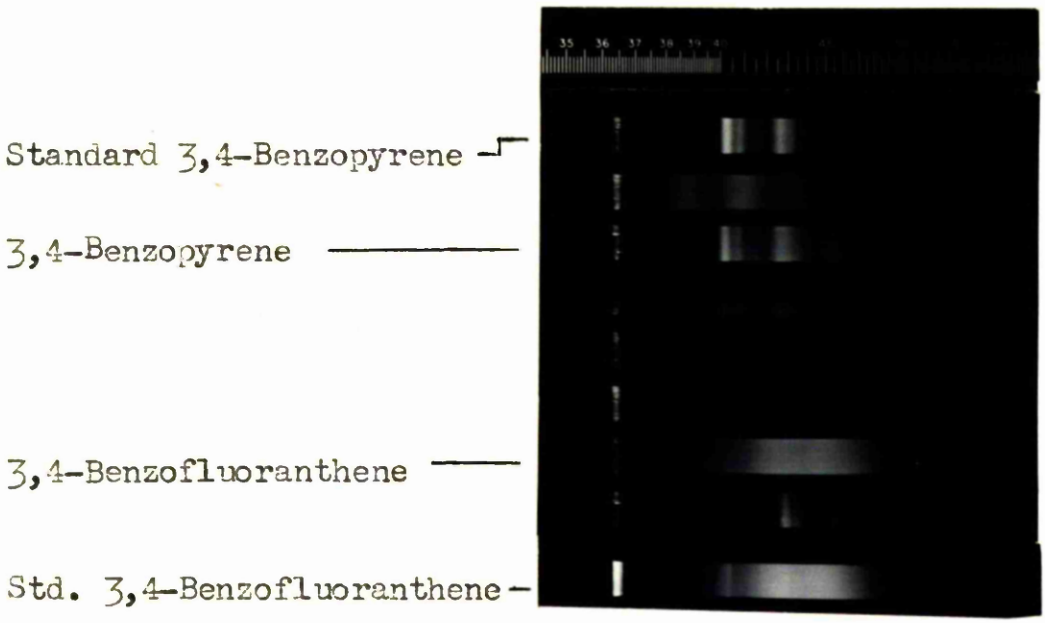
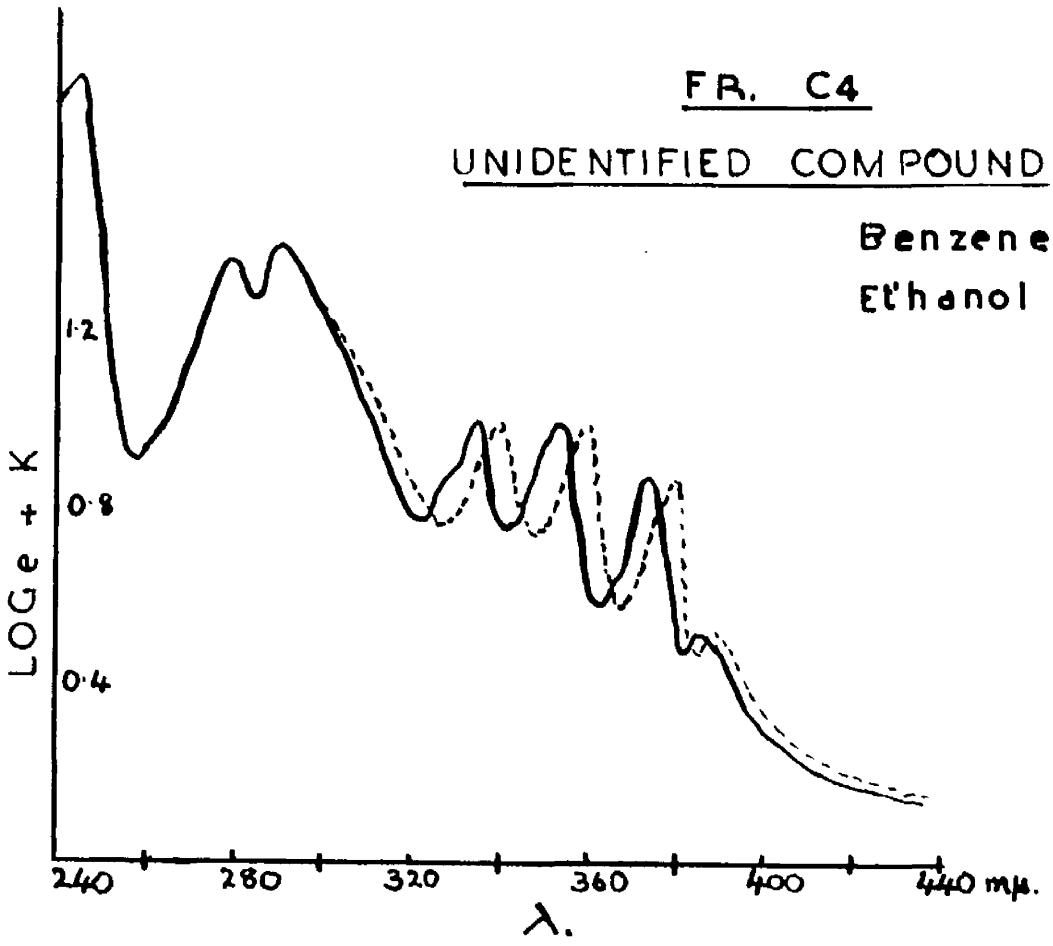


PLATE 6(a). Fluorescence spectra from the fractionation of petrol exhaust soot showing the occurrence of 3,4-Benzofluoranthene.

PETROL SOOT. Abs. Spectra.



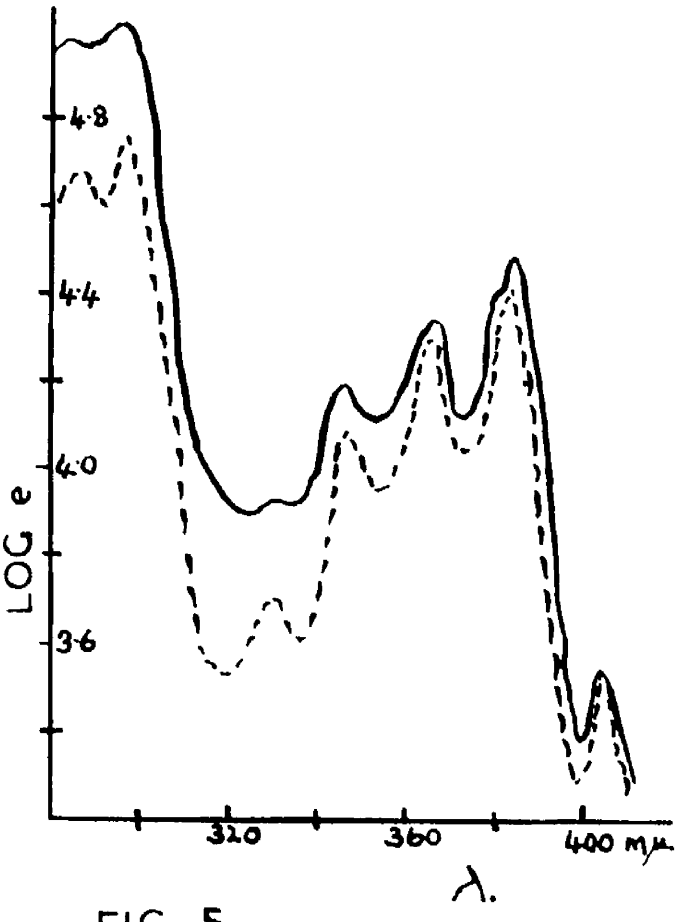
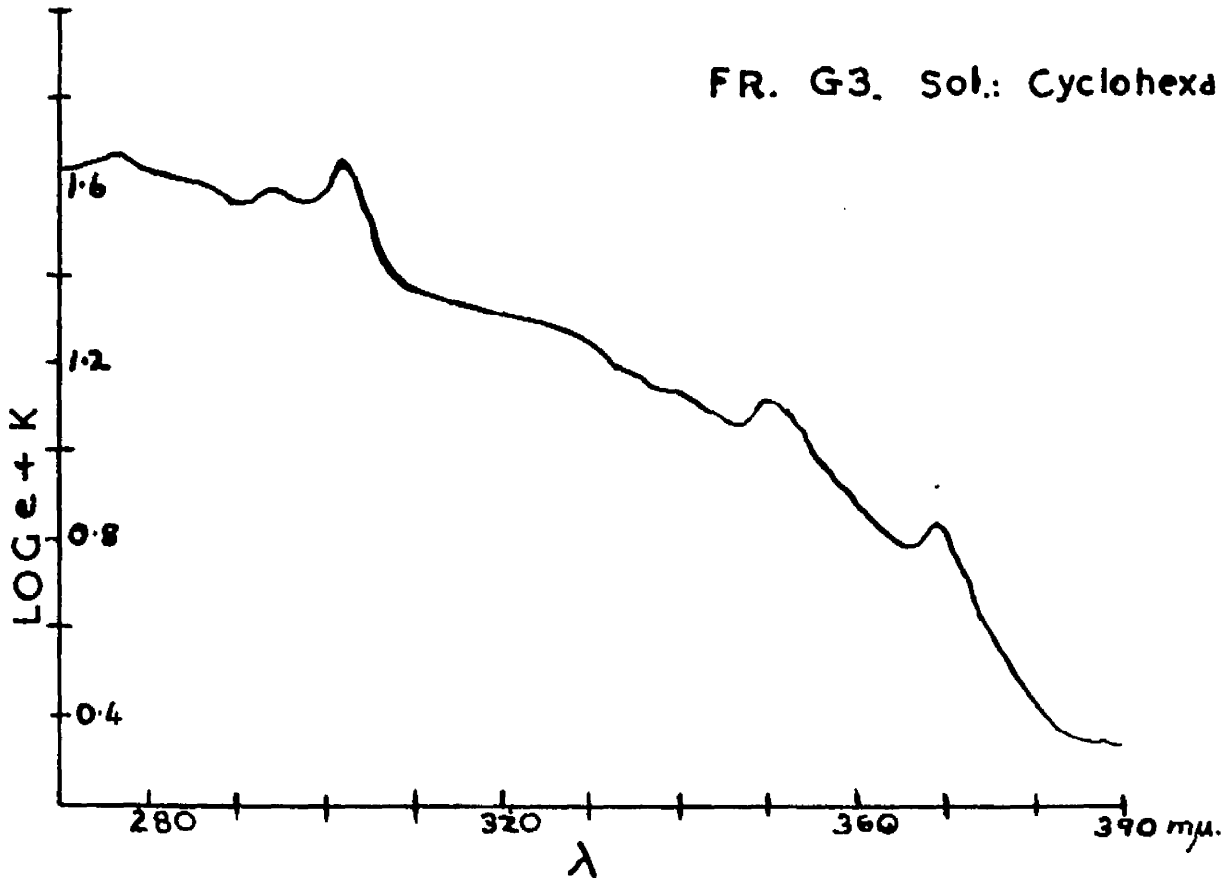


FIG 5.

PETROL SOOT Abs. Spectra



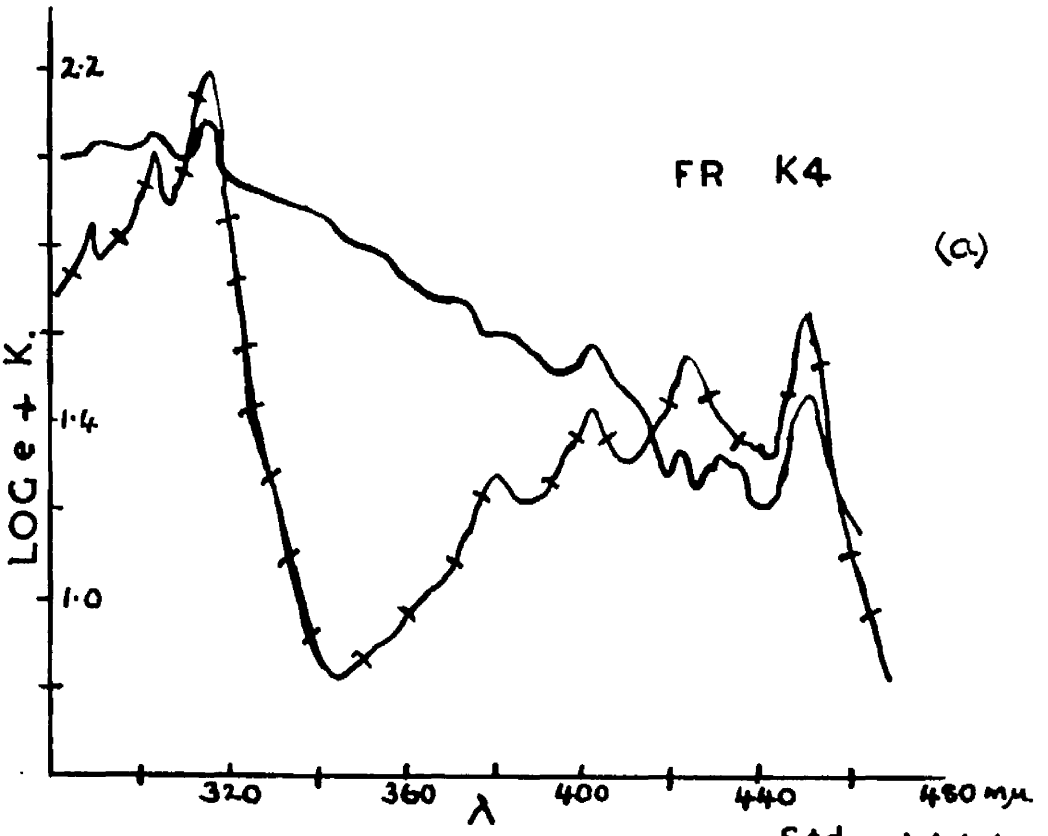
3,4-Benzofluoranthene ?

FIG 6.

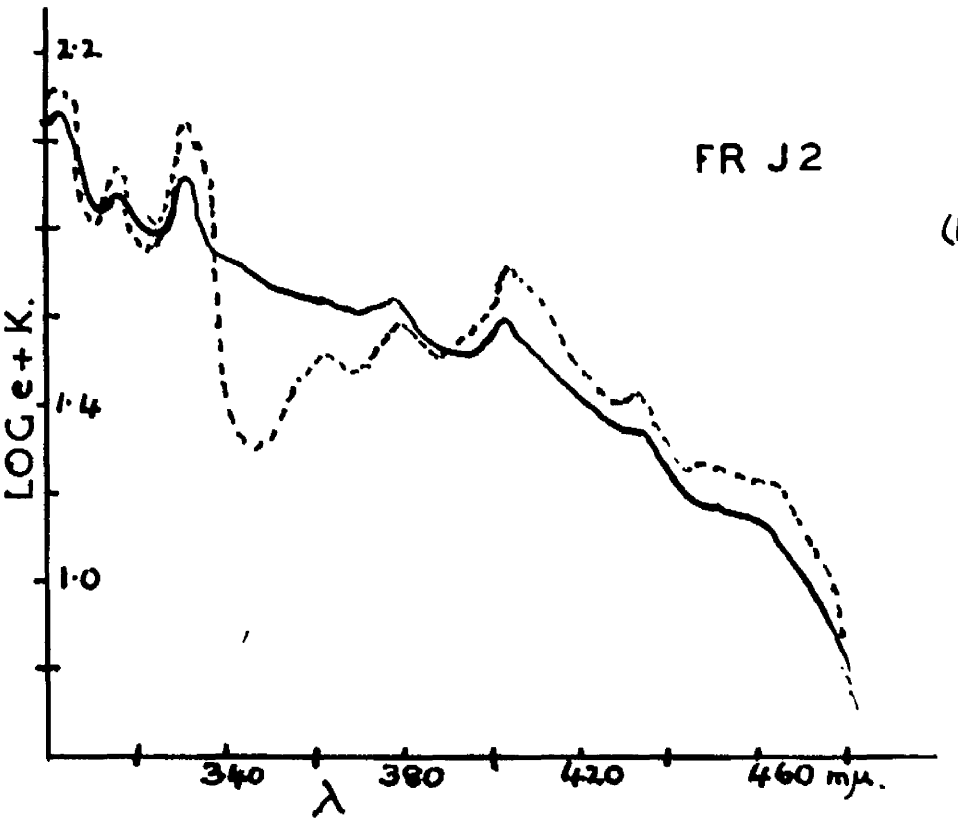
PETROL SOOT

Abs. Spectra

Sol.: Benzene.



3,4,8,9-Dibenzopyrene ?

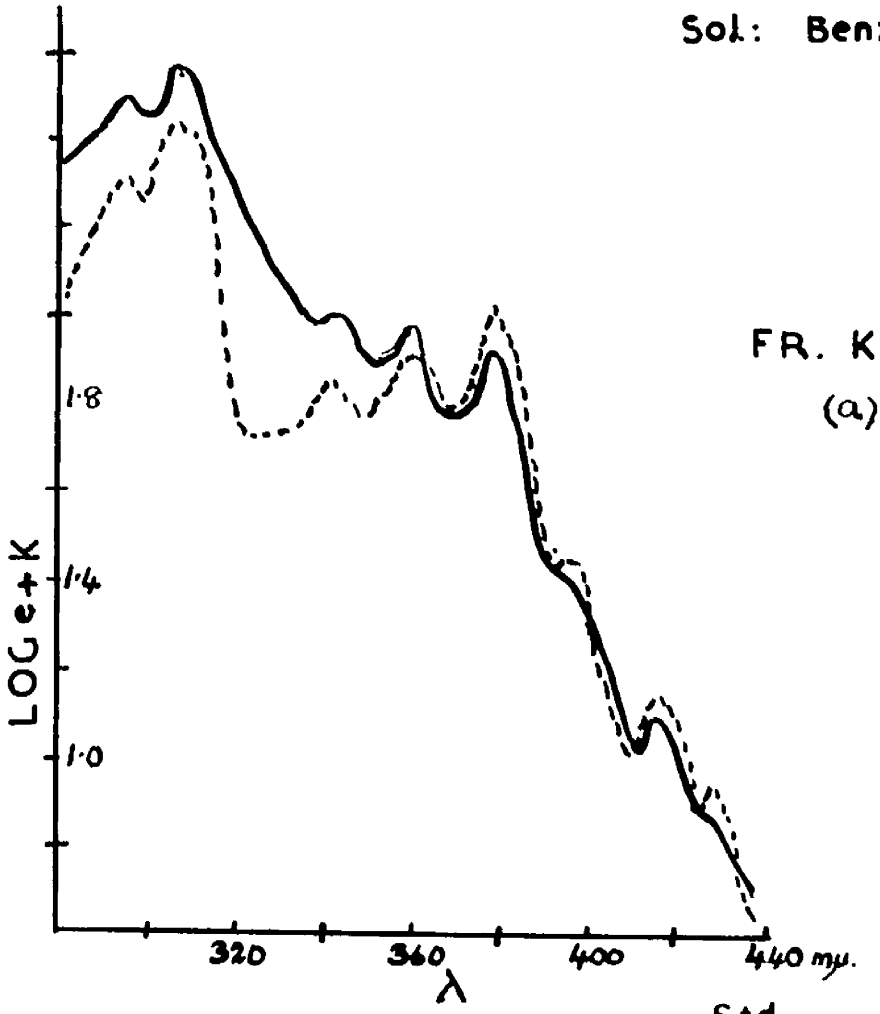


1,2,3,4-Dibenzopyrene.

FIG. 7.

PETROL SOOT. Abs. Spectra.

Sol: Benzene.



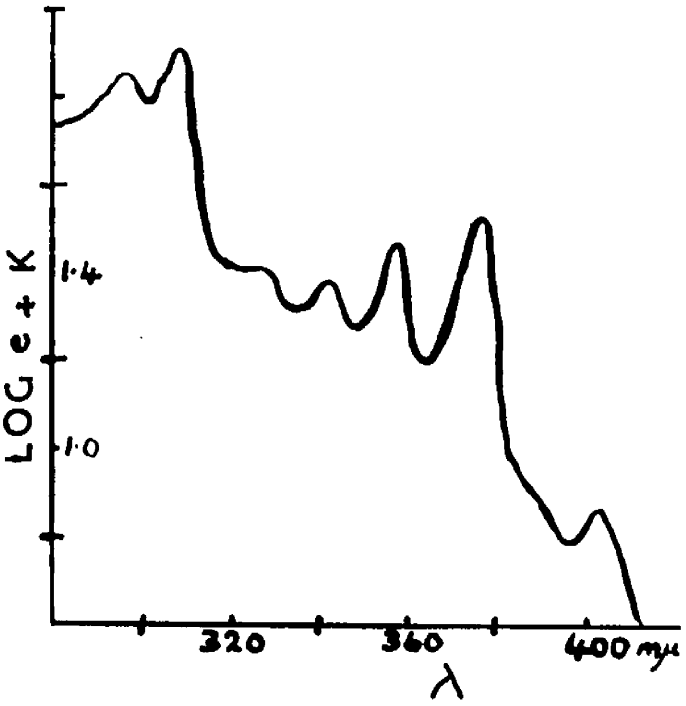
FR. K3.

(a)

Std. -----

Anal. ——

1,2,4,5-Dibenzopyrene



FR. K5.

(b)

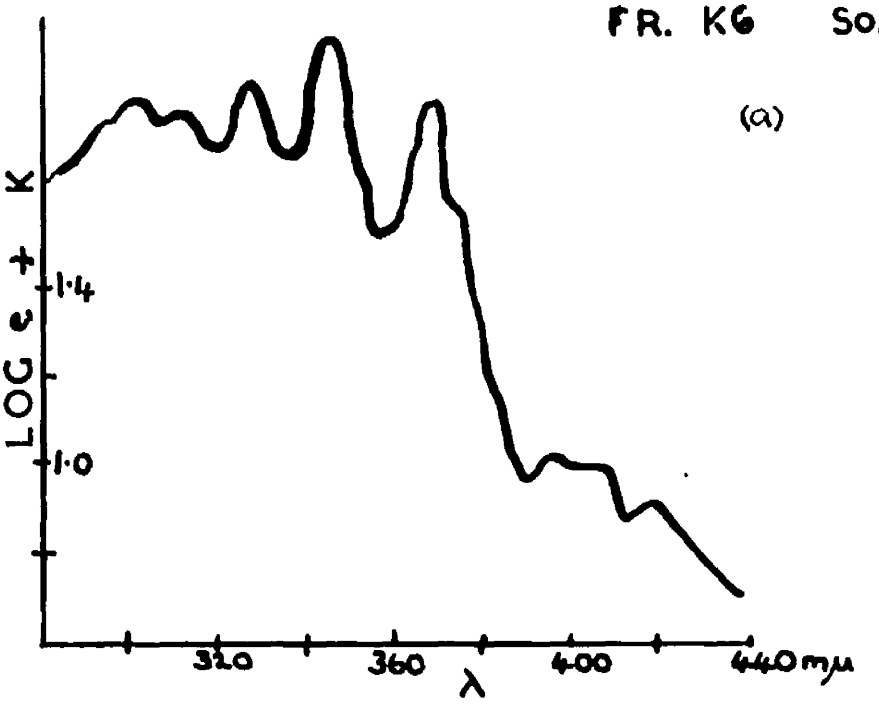
1,12,2,3-Dibenzoperylene

FIG. 8.

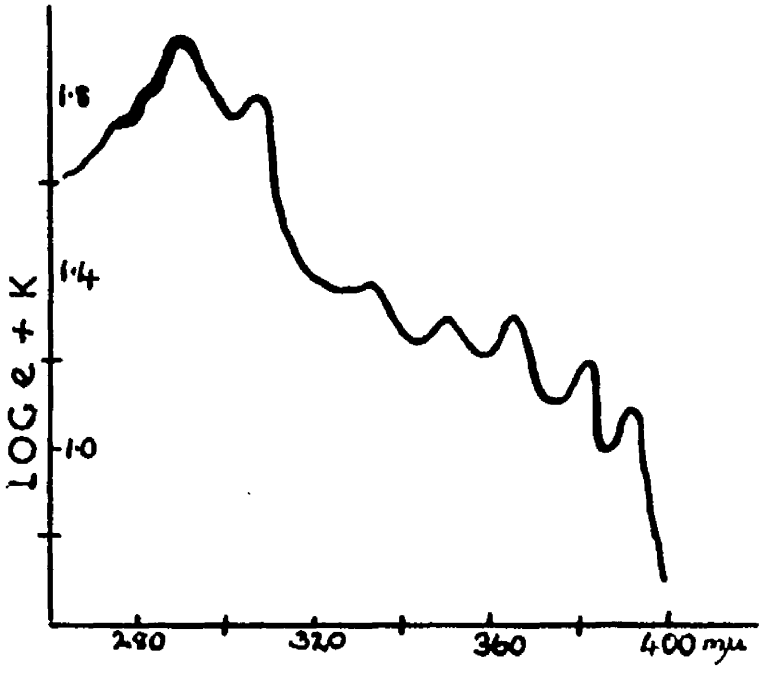
PETROL SOOT. Abs. Spectra

FR. K6 Sol.: Benzene.

(a)



Unidentified Compound.



FR. L2
Sol.: Cyclohexane.

(b)

Compound tentatively identified as 3,4-Benztetraphene

FIG. 9.

Section (D). Comparison of Vehicular Exhaust Soots and General Atmospheric Soot.

The vehicular exhaust soots consisted of oily particulate materials in contradistinction to the atmospheric soot which was gritty, reflecting the presence of much inorganic material, chiefly Silica. Both the exhaust soots had an acrid odour. This was particularly noteworthy in the case of the diesel soot. Organic solvent extracts retained much of the acrid smelling components and it is believed that such organic irritants are Aldehydes. (See Pattle et al, 1957).

In contrast to the vehicular exhaust soots, the atmospheric soot contained a relatively high percentage of resinous material - material extracted with acetone but insoluble in Petroleum-Ether. (See Table 8).

The aromatic hydrocarbons which occurred in highest concentration in the three soot samples appeared to be in the three to five condensed-ring range, i.e. from Anthracene and Phenanthrene to the Benzopyrenes.

The three soots had many components in common, including the well established pyrolytic products, Nephthalene, Anthracene and alkyl derivatives, Pyrene, Fluoranthene, Perylene, 1,2-Benzopyrene, 3,4-Benzopyrene, 1,12-Benzo-perylene, Coronene. 11,12-Benzofluoranthene, found in

highest concentration in diesel soot, was also detected in petrol and atmospheric soots. 3,4-Benzofluoranthene is tentatively identified in the petrol,diesel and atmospheric soots. Other unidentified compounds were found as common constituents. These are shown in Part Two, Section B(ii), where the compounds detected in the soot samples are tabulated side by side with the compounds detected in Cigarette main-stream smoke.

A notable difference between the exhaust soots and the general atmospheric soot was the apparent absence in the latter material of a fraction which was characterised by a green to light-blue fluorescence and which contained, among other components, the carcinogen 1,2,3,4-Dibenzopyrene. A similar finding was made with regard to Pentaphene and the Dibenzotetracenes.

Table 8 records the concentrations obtained for some of the compounds detected in the present analysis. The concentrations refer to parts per million of free carbon. Table 7 records the composition of the soots with regard to Acetone and Petroleum-Ether Extracts, free carbon and ash, as well as the ratios of Acetone and Petroleum-Ether extracted material and oil to free carbon.

Table 7

% by weight of whole soot.	Atmospheric Soot.	Diesel Exhaust Soot.	Petrol Exhaust Soot.
Acetone Extract	35.7	36.9	77.7
Petroleum-Ether Extract.	5.6	36.4	77.6
Oil	-	17.4	20.3
Free Carbon	34.1	59.7	22.1
Ash	27.0	0.9	-
Ratio			
Acetone Extract/ Free Carbon.	1.06	0.60	3.50
Petroleum-Ether/ Free Carbon.	0.17	0.58	3.49
Oil/Free Carbon.		0.29	0.92

Table 8

Concentration levels of hydrocarbons from Soots, in p.p.m. free Carbon.

Compound.	Atmospheric Soot.	Diesel Exhaust Soot.	Petrol Exhaust Soot.
Anthracene	215	60	385
Pyrene	650	820	440
1,2-Benzanthracene	70	56	180
3,4-Benzopyrene	380	20	1570
1,2,3,4,-Dibenzo-pyrene.	-	14	22
11,12-Benzofluor-anthene.	20	84	48
1,2,4,5-Dibenzopyrene	-	-	10

Addendum.

Since production of the present thesis began, a sample of pure crystalline 3,4-Benzofluoranthene was obtained (source: Dr. D.D. Hoffmann, Sloan-Kettering Inst., New York, U.S.A.).

The compound showed a brilliant blue fluorescence in cyclohexane. The absorption maxima given by the compound in this solvent were, 368,350,338,301,294,289,276 and 256 mμ. The fluorescence spectrum did not reveal any striking or discrete bands, and consisted of a region of fluorescence absorption commencing at 396 mμ, with two bands of elevated intensity having maxima at 428 and 450 mμ.

Both absorption and fluorescence spectra of the pure 3,4-Benzofluoranthene were comparable to spectra given by fractions from Petrol and Diesel exhaust soots and atmospheric soot obtained in the present investigations where 3,4-Benzofluoranthene was tentatively identified by U.V. absorption analysis.

This identification has now been substantiated.

Fluorescence spectra depicting the occurrence of 3,4-Benzofluoranthene in Petrol exhaust soot are now shown (Plate 6a), while the fluorescence spectrum of 3,4-Benzofluoranthene from Diesel exhaust soot with the standard for comparison, is included in Plate 9.

The spectra were not included in Plates 4,5 and 6.

PART TWO. INVESTIGATION OF CIGARETTE SMOKE

General Plan of Presentation

Part Two is divided into three main sections, A, B, and C. Section A is a general section and deals with the temperatures recorded during experimental smoking, comparing them with the temperatures recorded during human smoking.

Section B is divided into four sub-sections, B(i) and B(ii), B(iii) and B(iv). B(i) describes the investigation of cigarette main-stream smoke for the presence of aromatic hydrocarbons; B(ii) draws a comparison between the compounds found in the cigarette smoke and those found in general atmospheric, diesel and petrol exhaust soots; B(iii) describes the fate of a known quantity of a polycyclic hydrocarbon applied to cigarettes prior to smoking and subsequently subjected to the smoking process. In Section B(iv) is briefly described preliminary work on the composition of exhaled smoke as a possible source of atmospheric pollution and as a possible correction factor in the quantitation of any inspired smoke hazard.

Section C is divided into two sub-sections, C(i) and C(ii). C(i) deals with the investigation by the electron

paramagnetic resonance method of free radicals produced in cigarette smoke. C(ii) describes the reaction of cigarette smoke solutions with the stable free radical ∝∝'-Diphenyl-β-Picryl Hydrazyl and the detection of light-sensitive components in cigarette smoke.

Section (A). Experimental smoking temperatures

The primacy of combustion temperatures in the production of polycyclic aromatic hydrocarbons by pyrolysis has been attested ever since the pioneer work of Kennaway and his co-workers. Arising out of this work, it was considered imperative in the present studies, that experimentally produced cigarette smoke should approximate in composition - not only with respect to aromatic hydrocarbons, but also possibly other constituents - to cigarette smoke as produced in the human habit. Therefore the temperatures achieved in the experimentally smoked cigarette were compared with those achieved in the cigarette smoked by a human subject. A satisfactory correspondence was observed.

The Iron-Constantan thermocouple was both inserted laterally (at right angles to the axis of the cigarette) and threaded lengthwise in the cigarette. For temperature measurements in the cigarettes smoked by a human subject -

a habitual cigarette smoker performing, apparently, normally - the thermocouple was inserted laterally.

The quiescent temperature, i.e. the temperature recorded in the burning tip when air was not being drawn through the cigarette was found to vary between 580°C and 650°C. The average temperature of twenty recordings was 640°C. An average quiescent temperature of 610°C was found during human smoking.

Temperatures recorded in the burning coal of experimentally smoked cigarettes during draws of 2 to 4 seconds duration were found to vary between 730°C and 800°C. The average of twenty recordings was 750°C. Values approaching 800°C were regularly encountered as the cigarette diminished in length and resistance to uniform suction became less. The suction temperatures recorded during normal human smoking varied from 700°C to 800°C. The average of twenty recordings was 770°C.

The temperature of the main-stream smoke did not exceed 30°C as the cigarette was smoked to a stub length of 2 cms. A steep temperature gradient was noted. The temperature recorded in the cigarette smoke stream 0•5 cms from the burning tip during suction was between 900 - 1000°C.

While a summary of the above data was in Press (B.E.C.C. Ann. Rep. 1956, p 277) an article by Harlow appeared

(Science 123, 226, 1956) giving values in temperature profiles throughout cigarettes which corresponded very closely with the above values.

Table 9 shows the temperatures recorded by various workers during experimental cigarette smoking.

Table 9

Temperatures recorded in the cigarette during experimental cigarette smoking by various workers.

	Quiescent temp.°C	Suction temp.°C
Commins, Cooper and Lindsay (1954)	650	750
Seelkopf (1955)		700 - 795
Wynder, Graham and Croninger (1953)	313 - 599 Average: 430	Up to 966 Average: 692
Wynder and Wright (1957)	835 ± 30	884 ± 30
Ermala and Holsti (1956)	470	812 Average: 650
Harlow (1956)	746	774
Author (1955)	640	750

Section B(i). Aromatic polycyclic hydrocarbons in Cigarette main-stream smoke.

An examination of Hartwell's "Survey of compounds which have been tested for carcinogenic activity" (U.S. Public Health Service, 1951) and Supplement 1 of the same volume (1957), shows that, with rare exceptions - such as β-Naphthylamine - the carcinogenic members of the aromatic polycyclic hydrocarbon class, lie in that range of compounds which contain from three to six condensed benzene nuclei.

In an early paper (Nature 177, 630, 1956) mention was made of the detection in initial chromatographic eluates of Azulene and the compounds detected by Cooper and Lindsay (Bri.J. Can 9, 304,1955) Naphthalene, Acenaphthalene, and Anthracene and Pyrene. In the work about to be described, the identification of more strongly absorbed compounds which would lie within the carcinogenic range, was desired. Even though 3,4-Benzopyrene had been detected and estimated (Lyons, loc. cit.) the possibility was considered that other carcinogens of the polycyclic hydrocarbon class may be present. It will be recalled from the "Introduction" that evidence from chemical and biological investigations of other pyrolytic materials proves the occurrence of carcinogenic agents in fractions which precede and succeed 3,4-Benzopyrene on the chromatographic column.

The neutral aromatic tar fraction obtained from smoking 500 cigarettes weighed 4·40 g. This was chromatographed on a column of Alumina, using Petroleum-Ether as eluent initially. When what was expected to be Fluoranthene, as judged by that compound's behaviour on a parallel control column, was about to be eluted, 3% Acetone was incorporated into the eluent.

The appearance of the column showed a dark brown zone extending about 3 cms down from the top, which extended into a 0·5 cm tan-coloured band which had a bright blue-yellow fluorescence. Immediately below this band and extending about 1.5 cms down the column was a yellowish non-fluorescent zone which merged into a dull violet zone 0·5 cms in length. Below this zone blue-violet to blue areas extended down the length of the column without marked banding. To effect this degree of development 1·5 l of solvent had been used.

From the position of the 3,4-Benzopyrene band on the control column, the smoke tar Benzopyrene was expected to exist in the lower part of the dull violet zone, about 7 cms from the top of the column. The preceding fluorescent material was eluted with approximately 200 ml solvent Petroleum-Ether containing 3-5% Acetone, and designated Fraction A. The Benzopyrene fraction (Fraction B) was eluted with 200 ml solvent (Petroleum-Ether containing

5-10% Acetone). Two further fractions were eluted, Fraction with 200 ml of Petroleum-Ether containing 10-20% Acetone and Fraction D with 200 ml of Petroleum-Ether containing 20-40% Acetone. At this stage elution was terminated. The brown resinous material which originally had been absorbed in the uppermost part of the column, now extended throughout its length. The trickle of light-blue fluorescent material which could still be eluted from the column did not possess spectral features in the U.V. range 260 - 460 mμ. Fractions C and D were deep yellow-orange in colour and had a pleasant aromatic odour.

Following removal of the mixed solvent, each of the four main fractions was chromatographed on smaller columns of Alumina using Petroleum-Ether alone as solvent for Fraction A and Petroleum-Ether, containing 0-5% Acetone for fraction B, 0-8% Acetone for fraction C and 0-20% Acetone for fraction D. On the basis of fluorescence inspection of the columns in U.V. light, 4 sub-fractions were obtained from fraction A, and 10 sub-fractions from fraction B. As little discrete fluorescence zoning was apparent in the fractionation of C and D, cuts of approximately 100 ml were taken. The chromatography in each case was terminated when

little fluorescent material was appearing in the eluates. In all 44 sub-fractions were collected which appeared straw-coloured to orange in colour, the more strongly adsorbed fractions, in general, being most deeply coloured. The 44 sub-fractions were each passaged through columns of Silica Gel where some removal of the coloured contaminants occurred. For the strongly adsorbed components, a Petroleum-Ether/Ether solvent system was used. The fractions were screened by fluorescence spectrography and absorption spectrophotometry. All fractions showed considerable background absorption, yet some band systems were noticed, among them that of 3,4-Benzopyrene in a B sub-fraction and also in a C sub-fraction. Overlapping due to tailing had obviously occurred.

The sub-fractions were regrouped into 20 fractions, which were again chromatographed. In all repetitative chromatography yielded over a hundred fractions, which were screened by the fluorescence and absorption methods. Similar fractions were combined. In many fractions barely discernible spectral features, insufficient for any characterisation of compounds present, were encountered. Some fractions showing occasional small absorption peaks, occurred in such minute concentration as to render further purification impossible.

In fraction A the following compounds were detected. A light blue fluorescent eluate which consisted largely of Fluoranthene with the suggestion of 3-Methylpyrene (possessing peaks at 338 and 277 mμ) was followed by a violet-fluorescent eluate which showed the presence of 1,2-Benzanthracene and Chrysene. A further violet fluorescent eluate in this chromatographic region possessed the absorption features of 1,2-Benzofluorene, while a preceding eluate showed absorption features suggestive of 3,4-Benzophenanthrene with maxima at 372 and 280 mμ. A light blue fluorescent zone followed which contained 1,2-Benzopyrene and Perylene (which have been found closely associated in the various investigations).

In the 1,2-Benzopyrene eluates a compound with a main fluorescence maximum at 381 mμ was obtained. It had inflexio: points at 382,358 and 347 mμ, in cyclohexane.

As mentioned above, 3,4-Benzopyrene was detected in Fraction B (see Plate 8). It was preceded by 1,2-Benzopyrene and Perylene. Chromatographic purification of the 3,4-Benzopyrene eluate itself revealed the presence of two preceding spectrographically different components. The first had 1,2-Benzanthracene characteristics but exhibited a slight

bathochromic shift. Absorption analysis revealed the presence of a mixture of substituted 1,2-Benzanthracenes (see Fig. 10a). Small peaks were observed at 387, 360, 352, ~345, ~336, 330, 316, 303 and 280 (highest absorption peak) mμ. 1,2-Benzanthracene (standard) had peaks at 384, 368, 340, 328, 316, 300, 287 and 277 mμ. The fraction was considered as likely to be a mixture of 5,6 and 6,7-Cyclopentano - 1,2-Benzanthracene, as the absorption maxima closely fitted those of the standard compounds (see Maynoord and Roe, 1955). It was noted that Bonnet & Neukomm (1956) obtained spectrophotometric evidence for the presence of both these compounds in cigarette smoke tar.

Succeeding this eluate was a compound which showed a marked similarity to 3,4-Benzopyrene spectrographically and spectrophotometrically. Absorption peaks were obtained at 405, 387, 360, 342, 320, 306 and 292 mμ.

Following the 3,4-Benzopyrene eluates, eluates containing 1,12-Benzoperylene were obtained.

In the terminal eluate of this fraction definite spectrophotometric evidence for the presence of Pentaphene

was observed, absorption maxima at 423,399,359,317 and 306 mμ having been obtained.

Following the main 3,4-Benzopyrene fraction eluates were obtained which contained 1,12-Benzoperylene and Anthanthrene (in trace amounts) as well as a compound which had fluorescence maxima at 388,412 and 435 mμ and which seemed identical with a component of atmospheric soot. The identity of the latter has not been established. Blue-violet fluorescent components were next obtained which had fluorescence maxima at 413 and 437 mμ and 405 and 430 mμ. The former was observed in Petrol Exhaust and atmospheric soots. The latter had a fluorescence spectrum not unlike 3,4-Benzopyrene - slightly shifted towards the visible end of the spectrum. Therefore its fluorescence spectrum was compared with those of a series of Methylated 3,4-Benzopyrenes which had become available. The analytical material did not seem to correspond closely with any of the standard derivatives however, either in fluorescence or absorption. Absorption maxima were found at 406,391,380, 340,319,309,294,288 mμ. A blue-fluorescent eluate was next eluted which had a fluorescence spectrum similar to a Diesel Soot fraction which contained 1,2,9,10-Dibenzo-tetracene. However the absorption spectrum of the present

eluate showed corresponding peaks only in the U.V. end of the spectrum. The identity of the component(s) remains doubtful.

Chromatography of main fraction C yielded initial eluates of light yellow colour and blue fluorescence which did not seem to possess any notable spectral features. However, a later eluate was obtained which had a light green fluorescence. Purification of this eluate by alternate chromatography on Alumina and Silica Gel showed it to contain 1,2,3,4-Dibenzopyrene. The absorption and fluorescence spectra obtained showed all the features of the standard compound. The occurrance of the compound in the chromatographic sequence is shown in Plate 8. A blue-violet fluorescent eluate was next eluted which showed the characteristic fluorescence spectrum of 11,12-Benzofluoranthene. The absorption spectrum corresponded with that of the standard compound. This compound had been demonstrated previously in cigarette smoke (Lyons, 1956). The succeeding eluate contained traces of the same compound, on the fluorescence spectrum of which was superimposed another system, beginning at 450 mμ. The main absorption peaks of 3,4,8,9-Dibenzopyrene could be distinguished. A control chromatogram containing the two standard compounds verified the chromatographic sequence and mobility.

Eluates having a dull violet fluorescence were next obtained, possessing fluorescence bands at 343,352 and 363; 378,390,400; and 387,390,397,411,421 mμ. The latter two eluates showed absorption characteristics suggestive of the dibenzofluorenes. The absorption spectrum of the second compound is presented. It is tentatively identified as 1,2,7,8-Dibenzofluorene.

Chromatography of main-fraction D yielded five eluates which had banded fluorescence spectra. The first eluate, wit fluorescence bands at 385 and 407 mμ was present in extremely low concentration. The same system was observed in Petrol Exhaust Soot (Fr. L3). The next eluate had a violet fluorescence and had fluorescence maxima at 397,422 and 450 mμ. The same compound was found to occur in Atmospheric Soot (Fr. H1). The next eluate contained a compound which was also detected in both atmospheric and diesel soots (Fractions H2 and F5 respectively). It possessed a sharp fluted fluorescence band at 412 mμ and a broader band at 435 mμ. It possessed absorption maxima in Benzene at 412, 388,360, 318,298 mμ. A violet fluorescent component was next eluted which had an Anthracene-like system of three broad fluorescence, the third band of which was very faint. Its absorption spectrum resembled closely that of 1,2,3,4, 5,6- Tribensanthracene. The last eluate in this fraction

possessed rather sharp fluorescence bands at 442 and 472 mμ. This compound was also found in atmospheric soot (Fraction H3). It possessed some absorption characterisitics suggestive of 1,2,3,4-Dibenzotetracene (Anthracene 2',3',-9,10-Phenanthrene).

The compounds detected with relevant spectral features are shown in Table 10. Only compounds detected in the above investigation are tabulated. Anthracene, Pyrene and 3,4-Benzopyrene were estimated in a previous investigation. The quantities recovered per 100 cigarettes smoked were 12, 19 and 1•5 μg respectively. The concentration of 3,4-Benzopyrene estimated in the present investigation was 2•0 μg per 100 cigarettes while that of the 1,2,3,4,-Dibenzopyrene was 1•6 μg per 100 cigarettes smoked.

Table 10

Compounds detected in main-stream cigarette smoke neutral Aromatic Fraction

Fraction		Spectral Features(mμ)	Compound indicated
A1	(A	359,342,287,277	Fluoranthene
	(F	-	
	(A	328,277,464	3-Methylpyrene
	(F		
A2	(A	385,359,344,290,280	1,2-Benzanthracene
	(F	-	
	(A	266	Chrysene
	(F	-	
A3	A	372,280	3,4-Benzophanenthrene
	F	-	
A4	A	342,315,303,262	1,2-Benzofluorene
	F	-	
A5	A	388,366,332,317,304, 290,278.	1,2-Benzopyrene
	F	388	
	A	382, 258, 347.	
	F	381	
A6	A	437,411,386	Perylene
	F	437	
B1A	A	387,360,352, 345, 336,330,316,303,288.	5,6 and 6,7-Cyclopentono,1,2-Benzanthracene ?
	F	387,390,409,434.	
B1B	A	405,387,360,342,320, 306,292.	
	F	385,405,427,454.	

Table 10 (ctd.)

Fraction		Spectral Features (mμ)	Compound Indicated
B1C	A	404, 386, 365, 347, 298, 284.	3,4-Benzopyrene.
	F	404, 426, 454.	
B1D	(A	408, 388, 368, 303, 291.	1,12-Benzoperylene.
	(F	409, 431, 458.	
	(A	432, 408, 310	Anthanthrene.
	(F	-	
B1E	A	423, 399, 359, 317, 306.	Pentaphene
	F	"	
B2	A		
	F	388, 410, 435.	
B3	A	413, 400, 383, 362, 345, 317, 303.	
	F	413, 437.	
B4	A	406, 391, 380, 340, 319, 309, 294, 288.	
	F	405, 430.	
B5	A	435, 423, 396, 377, 368, 336, 325, 300.	
	F	434, 464.	
C1	A	462, 454, 433, 401, 381, 332, 317.	1,2,3,4-Dibenzopyrene.
	F	462.	
C2	A	401, 380, 360, 309, 296.	11,12-Benzofluoranthene.
	F	401, 427, 456.	
C3	A	451, 424, 401, 380, 360, 314, 310.	11,12-Benzofluoranthene and 3,4,8,9-Dibenzopyrene.
	F	401, 427, 456; 450.	
C4	A	343, 330, 315, 306, 288, 268.	
	F	343, 352, 363.	

Table 10 (ctd.)

Fraction		Spectral Features (mμ)	Compound indicated
C5	A	343,333,320,306,267,258.	
	F	378,390,400.	1,2,7,8-Dibenzo-fluorene.
C6	A	387,344,335,324,300, 288.	
	F	387,390,397,411,421.	
D1	A	385,363, 348,312,302.	
	F	385,407.	
D2	A	397.	
	F	297,422,450.	
D3	A	412, 396,384,364, 350,303,294,286.	
	F	412,435.	
D4	A	388,378,368,345,333, 318,384,290.	1,2,3,4,5,6-Tribenz Anthracene.
	F	388,412.	
D5	A	442,413, 318,306	1,2,3,4,-Dibenzo-tetracene?

The solvent used in the absorption spectrophotometry of fractions A1 to B1D inclusive is Cyclohexane. The absorption data for the remainder refer to Benzene as solvent.

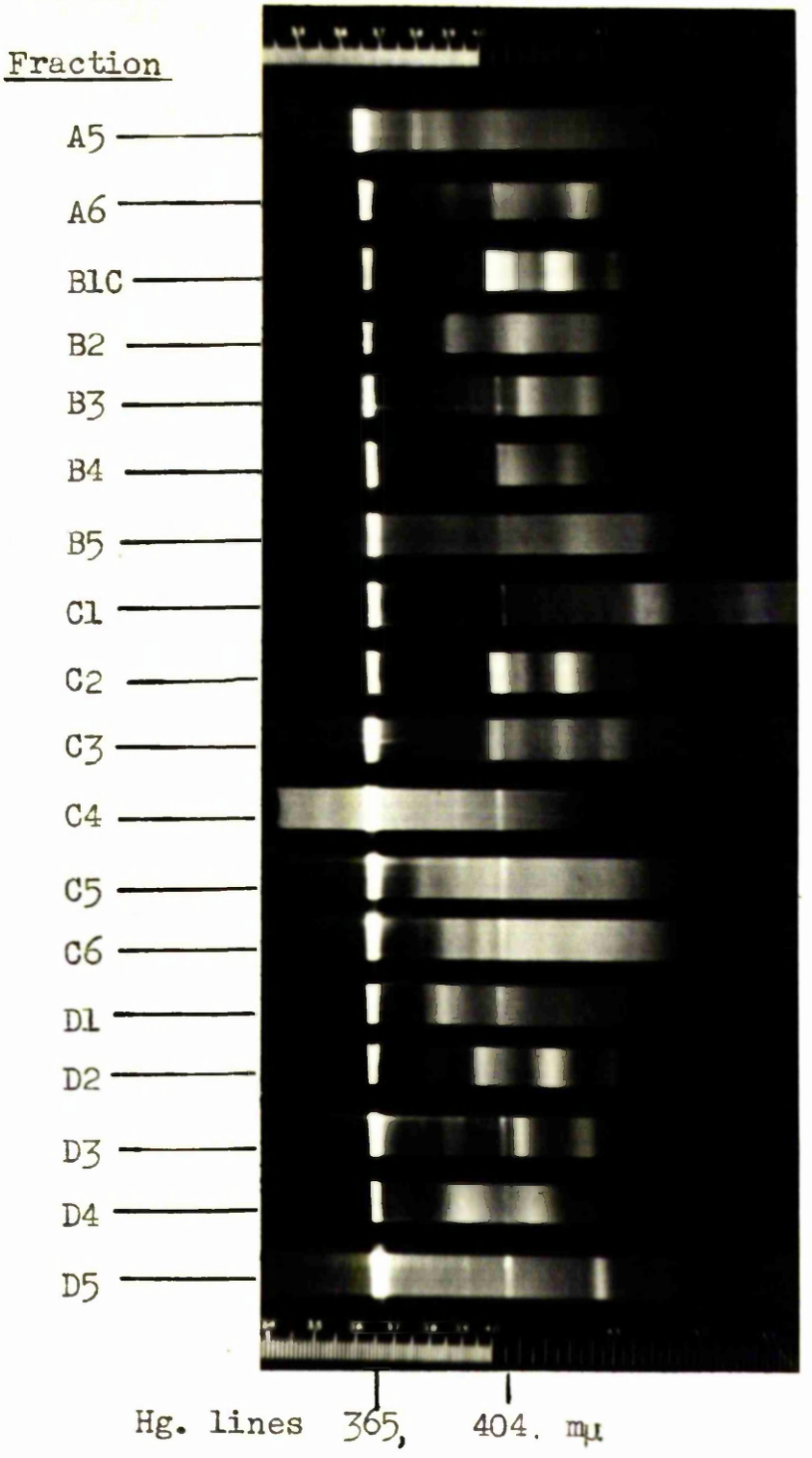


PLATE 7. Fluorescence Spectra of sub-fractions obtained from the neutral aromatic fraction of cigarette main-stream smoke.

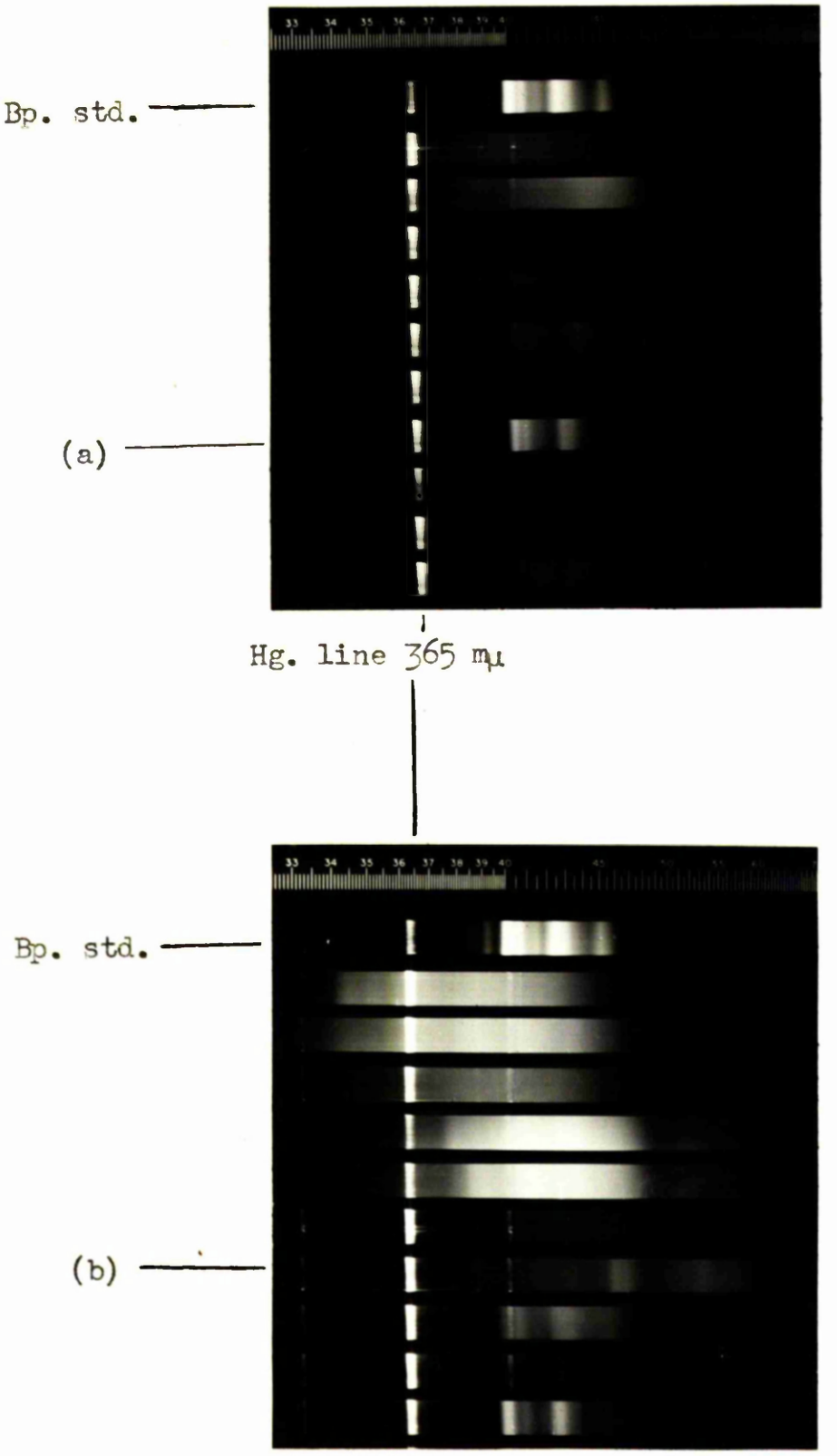


PLATE 8. Fluorescence Spectra from the fractionation of cigarette smoke, showing the occurrence of (a) 3,4-benzopyrene; (b) 1,2,3,4-dibenzopyrene.

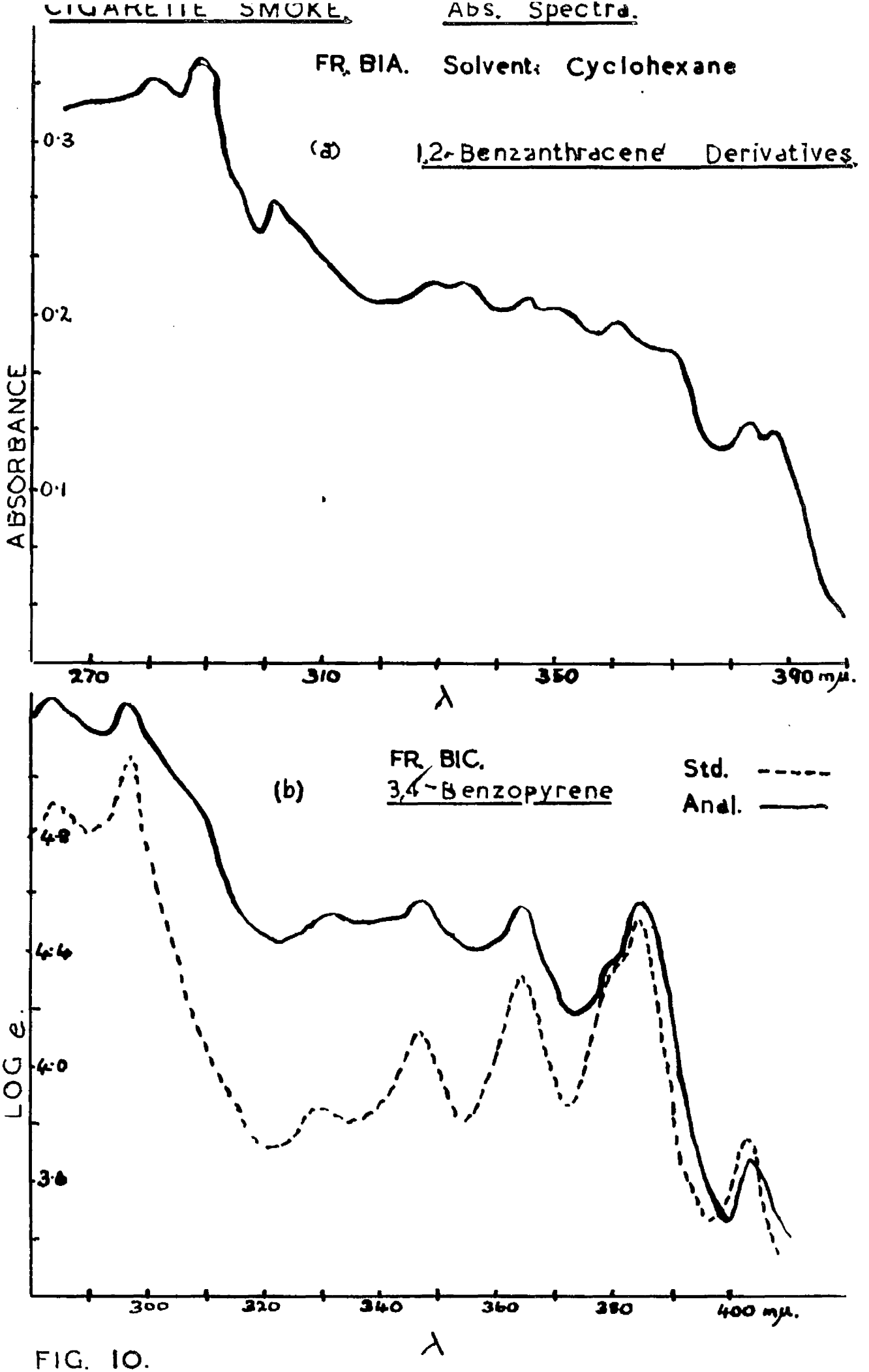
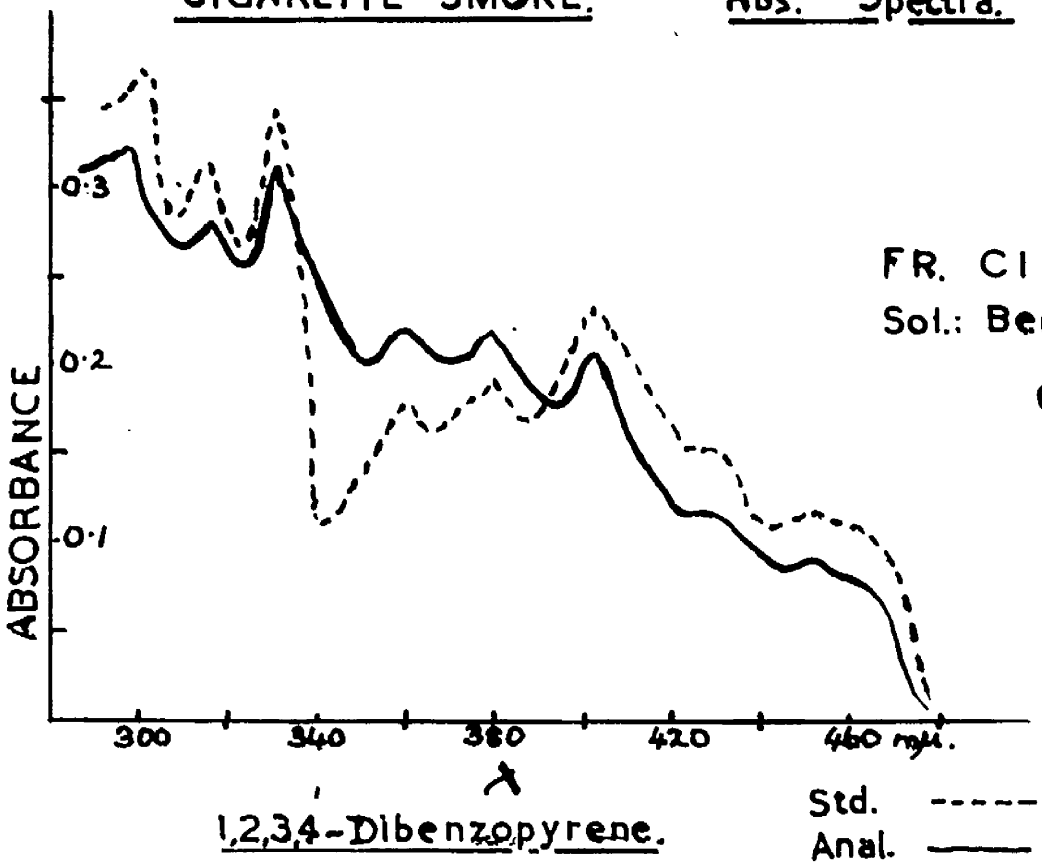
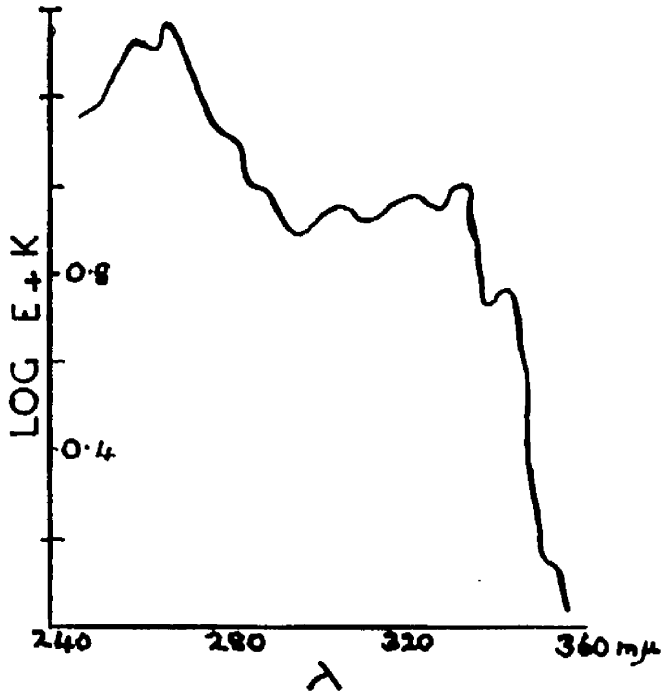

CIGARETTE SMOKE.
Abs. Spectra.
FR. BIA. Solvent: Cyclohexane
(a) 1,2-Benzanthracene Derivatives.
0·3
0·2
0·1
ABSORBANCE
270
310
350
390 mμ.
λ
FR. BIC.
(b) 3,4-Benzopyrene
Std. -----
Anal. ——
4·8
4·4
4·0
3·6
LOG e.
300
320
340
360
380
400 mμ.
λ

FIG. 10.

CIGARETTE SMOKE. Abs. Spectra.



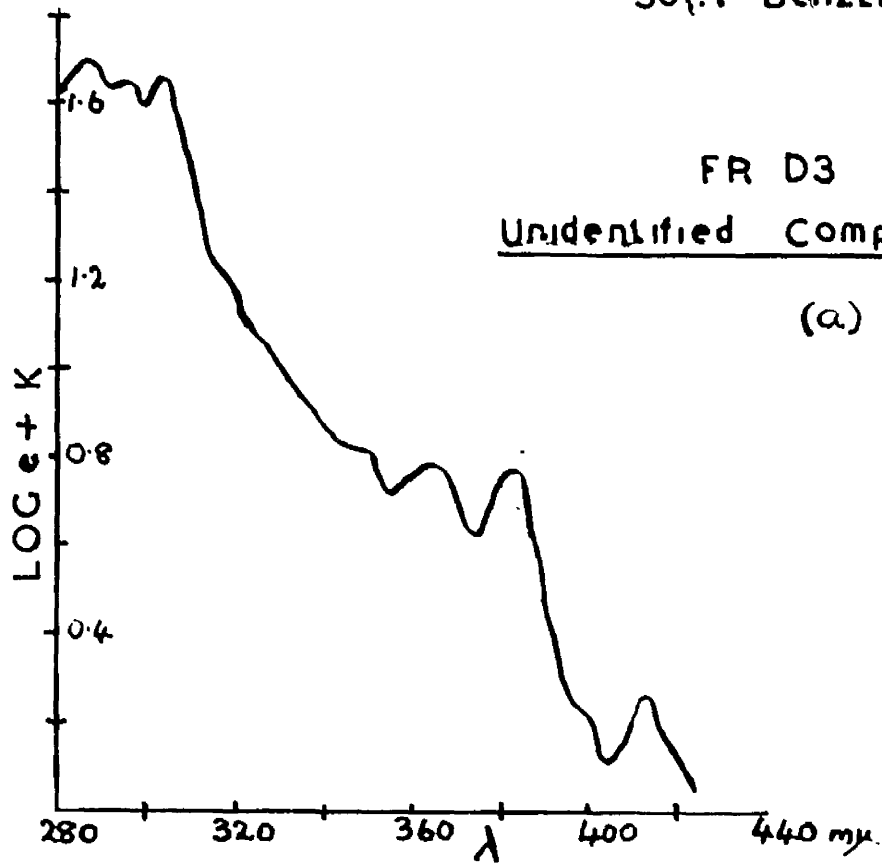
1,2,3,4-Dibenzopyrene.



1,2,7,8- Dibenzofluorene ?

FIG. 11.

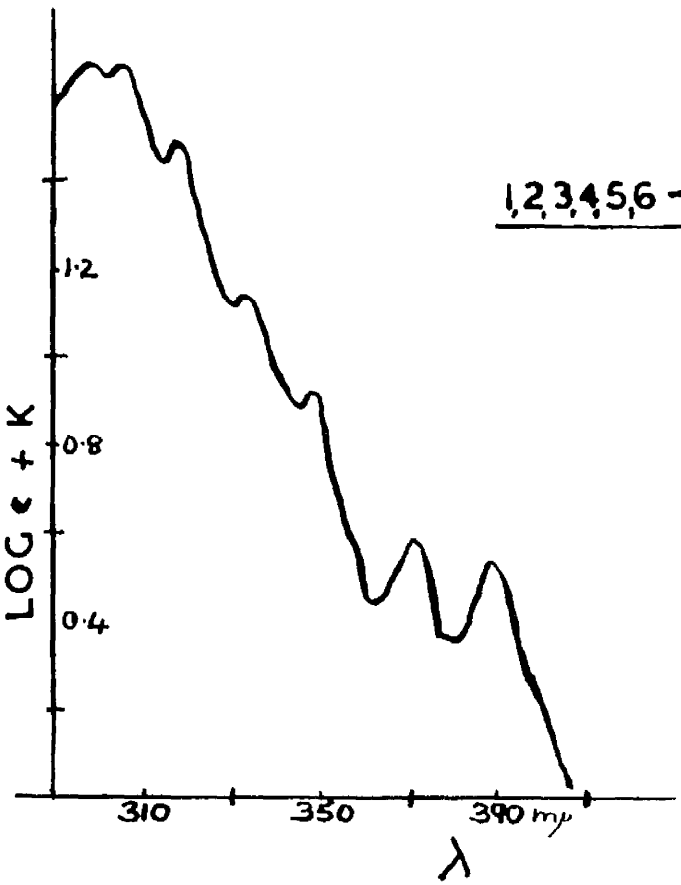
Sol.: Benzene.



FR D3

Unidentified Compound

(a)



FR D4

1,2,3,4,5,6 - Tribenzanthracene

(b)

FIG 12.

Section B (ii)

Comparison between Cigarette main-stream smoke and General Atmospheric, Diesel and Petrol Soots with respect to Aromatic hydrocarbons.

The following table (Table 11) lists the hydrocarbons detected in the present investigation in the cigarette smoke and the three soots. Compounds which, on the basis of their absorption and/or fluorescence spectra, are common to two or more of the source materials are tabulated on horizontal columns. The presence or absence of carcinogenic potency associated with the compounds, in so far as this quantity or property is known, is indicated. The compounds are indicated by their fraction number.

Table 11

Aromatic hydrocarbons[+] detected in main-stream Cigarette Smoke and general atmospheric, Diesel and Petrol Exhaust Soots: A comparison.

Compound	Cigarette smoke.	Atmospheric soot.	Diesel Soot	Petrol Soot.	Carcinogenic potency.
1. Azulene	Initial Eluates.	"	"	"	"
2. Naphthalene and simple derivatives.	Initial Eluates.	A1	A	A	"
3. Acenaphthylene.	"	A2	A	A	"
4. Anthracene.	Early Eluates.	A3	B1	B1	"
5. Phenanthrene.	" (?)	B1	B2	"	"
6. Anthracene derivative I.	"	"	"	B2	"
7. Anthracene derivative II.	"	B3	C2	B3	"
8. Anthracene derivative III.	"	"	"	B4	"
9. Pyrene.	Early Eluates	B2	C1	C1	"
10. Fluoranthene	A1	C1	C3	C2	"
11. 3-Methylpyrene.	A1	"	"	C3	"
12. "Orange Compound.	"	"	C4	C4	?
13 Anthracene derivative IV.	"	"	"	D1	
14. Anthracene derivative V.	"	"	"	D2	

Table 11 (ctd.)

Compound	Cigarette smoke.	Atmospheric soot.	Diesel Soot	Petrol Soot	Carcinogemic potency.
15. 1,2-Benzanthracene.	A2	C2	C5	E1	+
16. Chrysene	A2	C3	-	-	±
17. 3,4-Benzophenanthrene.	A3(?)	-	-	-	+ -
18. 1,2-Benzofluorene.	A4	-	-	-	-
19. 1,2-Benzopyrene.	A5	D1	D1	F1	±
20. Unknown	A5	?	-	-	
21. Unknown	?	C4	-	-	
22. Perylene	A6	D2	D2	F2	
23. Benzanthracene derivatives.	B1A	-	-	-	++
24. "3,4-Benzopyrene-like" (hydrogenated)?	B1B		D3	-	
25. 3,4-Benzopyrene.	B1C	D3	D4	G1	+++
26. 1,12-Benzoperylene.	B1D	D4	D5	G2	±
27. 3,4-Benzofluoranthene.	-	E1A	D6	G3	++ (?)
28. Anthanthrene	B1D	-	-	G4	-
29. Tetracene (Naphthracene)	-	-	-	H1	-
30. Pentaphene.	-	-	E1	H2	-

Table 11 (ctd.)

Compound.	Cigarette smoke.	Atmospheric soot.	Diesel Soot.	Petrol Spot.	Carcinogonic potency
31. Coronene.	B1E	-	E2	H3	-
32. Unknown.	B2	E1B	-	-	
33. Unknown	B3	E2	D7	K1	
34. Unknown	B4	-	-	L1	
35. Unknown	B5	E3	-	-	
36. Unknown	-	-	E3	J1	
37. 1,2,3,4-Dibenzopyrene.	C1	-	E4	J2	+++
38. 11,12-Benzofluoranthene.	C2	F3	F2	J3	-
39. 3,4,8,9-Dibenzopyrene.	C3	-	-	K4	+++
40. Unknown.	-	-	F1	-	
41. Unknown.	-	F1	-	-	
42. Unknown.	-	F2	-	-	
43. Unknown	-	F4	-	-	
44. 1,2,9,10-Dibenzotetracene	?	-	F3	J4	-
45. Unknown	-	-	-	K2	
46. 1,2,4,5-Dibenzopyrene.	-	-	-	K3	+++
47. Unknown	-	-	-	K4	

Table 11 (ctd.)

Compound.	Cigarette smoke.	Atmospheric soot.	Diesel Soot.	Petrol Soot.	Carcinogenic potency.
48. 1,12,2,3-Dibenzoperylene?	-	-	-	K5	
49. Unknown	-	-	-	K6	
50. Unknown	C4(?)	G1	-	-	
51. Unknown	-	G2	F4	-	
52. Dibenzofluorene.	C5	G3	-	-	+
53. Unknown	C6	-	-	-	
54. 3,4-Benzotetraphene.	-	-	-	L2	
55. Unknown	D1	-	-	L3	-
56. "	D2	H1	-	-	
57. "	D3	H2	F5	-	
58. 1,2,3,4,5,6,-Tribenzanthracene.	D4	-	-	-	-
59. 1,2,3,4,-Dibenzotetracene?	D5	H3	-	-	
60. Unknown	-	-	-	L4	
61. Unknown	-	H4	-	-	

+ The structural formulae of the main compounds identified are shown in the Appendix to the present thesis.

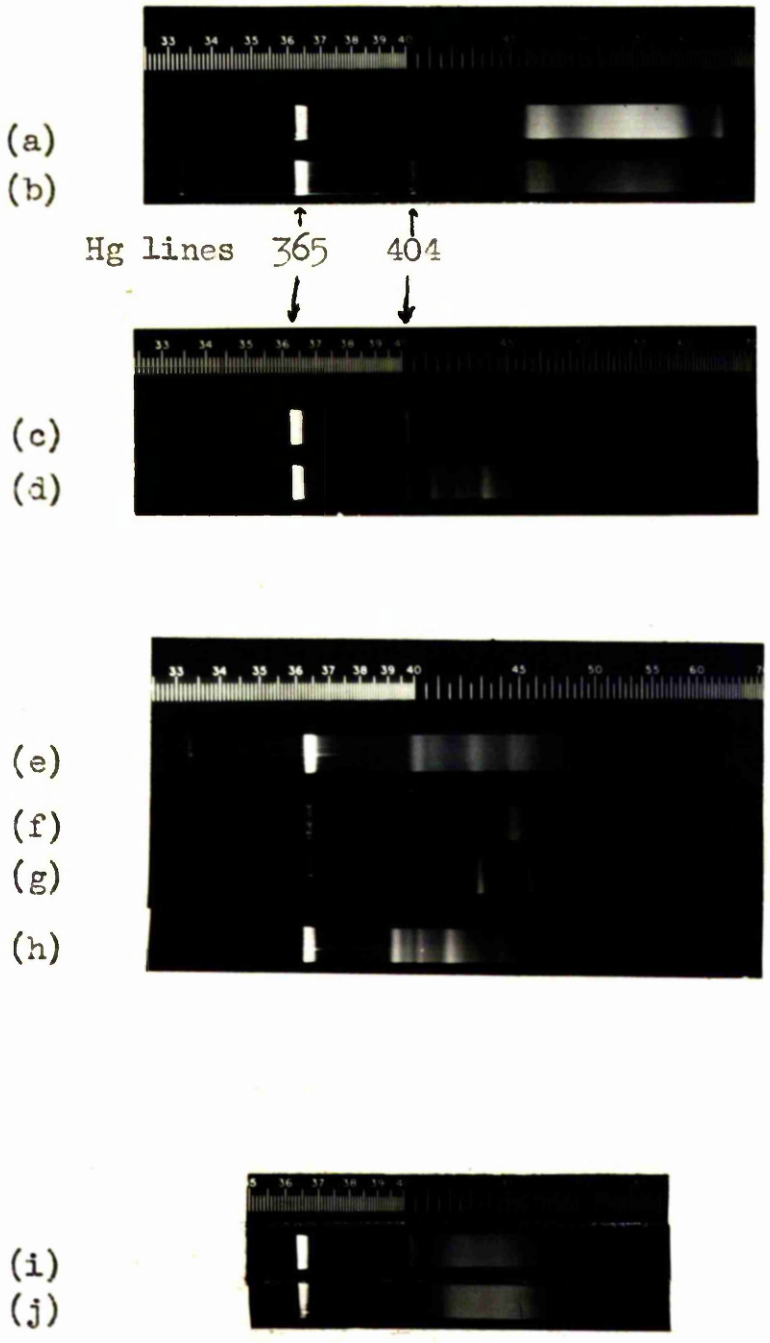


PLATE 9. Fluorescence Spectra of some carcinogenic hydrocarbons obtained in the course of the present investigations.

Explanation to Plate 9.

Explanation to Plate 9.

(a) Standard 1,2,3,4-Dibenzopyrene.

(b) 1,2,3,4-Dibenzopyrene from cigarette smoke.

(c) 1,2,4,5-Dibenzopyrene from petrol exhaust soot.

(d) Standard 1,2,4,5-Dibenzopyrene.

(e) Fraction from cigarette smoke containing 3,4,8,9-Dibenzopyrene.

(f) Standard 3,4,8,9-Dibenzopyrene.

(g) Standard 3,4,9,10-Dibenzopyrene (not found).

(h) Standard 1,2,6,7-Dibenzopyrene (not found).

(i) Standard 3,4-Benzofluoranthene.

(j) 3,4-Benzofluoranthene from diesel exhaust soot.

Section B (iii)

The fate of a known quantity of 3,4-Benzopyrene applied to cigarettes prior to smoking.

Quantities of 500 and 10 µg of 3,4-Benzopyrene in ethereal solution were applied to two lots of 50 cigarettes each by end-on immersion of the cigarettes. Care was taken in the applications that none of the added hydrocarbon soaked up to within 2 cm of the prospective stub-ends - the rod ends which were inserted in the smoking manifold. Smoking and collection of smoke products was carried out as described under "Methods" in the present thesis.

In the 500 µg application experiment, the 3,4-Benzopyrene was suspected by its mobility and fluorescence on the main-stream and side-stream smoke and stubs chromatograms, but not on the chromatogram of the ash extract. The latter exhibited one bright blue strongly adsorbed band and was completely different in appearance from the three other chromatograms. The main stream smoke and stubs chromatograms were very similar in appearance, while the side-stream chromatogram differed from them in having a disproportionately larger amount of light-blue fluorescent material of greater chromatographic mobility than 3,4-Benzopyrene.

Screening of fractions by the spectrographic and spectrophotometric methods showed the Benzopyrene to be

present in the main-stream and side-stream smoke and in the stubs. In the experiment where 10 µg of the hydrocarbon was applied, the Benzopyrene was detected in main-stream and side-stream smoke (in trace amounts in the latter). The percentages of Benzopyrene recovered in the different combustion fractions are shown in Table 12.

After making an allowance for a possible loss of up to 20% incurred in the chemical manipulation, these experiments demonstrate that considerable destruction of the added 3,4-Benzopyrene occurred. The presence in the side-stream of 3,4-Benzopyrene suggests that the compound may survive the high temperatures in the region of the burning tip and through volatilisation enter the general atmosphere. This further suggests that, as this compound (among many others) is formed in human smoking, the latter may contribute to the atmospheric pool of this carcinogen.

Table 12.

Percentage recovery of 3,4-Benzopyrene added to cigarettes before smoking in main-stream and side-stream smoke, butts and ash.

Amount of Benzo-pyrene added.	Main-Stream Smoke.	Side-Stream Smoke.	Stubs	Ash	Total
500 µg	20	4	4·5	-	28·5
10 µg	20	2	-	-	22·0

Section B (iv). Experiments on the composition of exhaled cigarette smoke.

A human subject smoked 20 cigarettes, exhaling the smoke into a Dreschel bottle containing fluorescent-free Benzene.

The benzene solution of smoke thus trapped had a light yellow colour, presented a cloudy appearance due to moisture and had, on drying with a little anhydrous Sodium Sulphate, a blue fluorescene.

A sample of this solution showed the presence of reducing substances - which were shown to be present in inspired (main-stream) smoke - by reacting with the stable free radical $\alpha\alpha'$-Diphenyl-β - Picrylhydrazyl. (See Section C).

The bulk of the benzene solution was extracted with dilute acid and alkali. The acid solution on concentration showed a grey precipitate with Silica-tungstic acid indicative of Alkaloids. A spot of the concentrated acid extract on filter paper stained brown on treatment with p.Aminobenzoic acid and Cyanogenbromide, indicative of Nicotine.

The neutral aromatic fraction was dissolved in Petroleum-Ether, layered on to a column of Alumina and chromatographed in parallel with a similar fraction of main-stream smoke.

The two chromatograms presented a striking similarity on development with Petroleum-Ether and later with Petroleum-Ether containing 2% Acetone.

A blue-fluorescent Petroleum-Ether eluate from the expired smoke chromatogram, suspected of containing Pyrene was rechromatographed on Silica Gel and Alumina. A U.V. absorption analysis of this fraction showed inflexion points at 372, 335 and 318 mμ in Cyclohexane indicative of Pyrene, as well as a region of maximum absorption from 288 to 275 mμ.

A rough estimate indicates that approximately 90% of inspired smoke is retained and 10% exhaled. There would not appear to be any selective retention on the part of the respiratory epithelium.

SECTION C. THE DETECTION AND INVESTIGATION OF FREE RADICALS IN CIGARETTE SMOKE.

Section C(1). Experiments using the Electron Paramagnetic Resonance Absorption Method

The experimental arrangement was as described in the "Methods" portion of the present thesis.

Thirty cigarettes were smoked with intermittent drawing as described and approximately 0·5 g condensate, which occupied a 6 cms length of the tube, was collected at the temperature of liquid oxygen, 90°K. On transference to the electron resonance spectrometer a signal with a "g" value typical of the free electron at 2· was obtained with this condensate (Fig. 16). On integration, the area traced out by the signal corresponded approximately with a standard free-radical sample, of concentration 10^{15} free electrons per g.

The condensate was expected to contain large amounts of solid carbon dioxide and ice. The presence of the former was shown by the evolution of gas when the tube was removed from the refrigerated cavity for heating at 60°C for about 10 minutes. The rest of the condensate on warming was found to separate into an aqueous and an organic layer. The latter

occupied a 1 cm length of the tube long, i.e. this quantity of organic material had been diluted six times in the original condensate.

The two layers in turn were examined for free radicals after cooling to 90°K. As might be expected, no free radicals could be detected in the aqueous layer. The organic phase however gave a signal approximately equivalent to the signal obtained from the whole condensate in which it was diluted by a factor of six. Therefore it would seem that the organic constituents of cigarette smoke condensate contain about 6×10^{15} free electrons per g or 6×10^{-8} moles per g. For more accurate measurements a small correction factor for density would have to be employed in the calculation. It has been shown above that the organic constituents after warming the condensate contain 10^{15} free electrons per g. Therefore a reduction in the concentration of free radicals by a factor of approximately six occurs, or, the cigarette smoke condensate contained unstable relatively short-lived free radicals at a concentration level of about 5×10^{15} free electrons per g.

The residual radicals that remained in the tar after warming were found to be highly stabilised and no diminution in their concentration was found after several days, even on

exposure to air. Limited chemical and chromatographic fractionation was carried out on these radicals.

For this cigarette smoke was trapped in a series of Dreschel bottles containing Benzene at room temperature. The total smoke solution was divided into 5 aliquots. The first portion was untreated and is referred to as 'whole tar'. The second portion was washed three times with water; the third with 2N·NaOH; the fourth with 2N·H_2SO_4 and the last portion with the acid and alkali to give the so-called neutral fraction The last three portions were each finally washed with water. The solvent from each of the five aliquots was removed under reduced pressure and the free radicals of the resulting tars estimated.

It was found that washing with water reduced the radical concentration of the original whole tar by 20%, washing with alkali by 50%; with acid by 57%; while washing with acid and alkali reduced the concentration by 72%. It seems, therefore, that a number of different species of free radicals exist in the stable group.

A similar benzene solution of cigarette smoke was prepared for a chromatographic fractionation. Fresh Spence 100-200 mesh Alumina was mixed into the solution until all the liquid had been taken up by the adsorbent. This was carried out in the

dark. Residual benzene was allowed to evaporate off in air and the resulting Alumina with adsorbed tar was filled into a column which was subsequently shielded from the light. The column was then washed with Hexane until no further material was being eluted. The same procedure was carried out with Benzene and Acetone consecutively. The solvents were evaporated from the three eluates and the free radical content of the resulting tars estimated.

No free radical concentration could be detected in the Hexane fraction while the Benzene and Acetone fractions in turn possessed concentrations 35% and 50% that of the original tar. This showed that the major portion of the radicals could be desorbed from Alumina with a facility which increased as the polarity of the solvent was increased.

This chromatographic behaviour would suggest that a high percentage of the radicals consist of ring clusters of a complexity increasing from structures of 4 or 5 condensed nuclei. An analogy may be drawn with the free radicals of carbons (Austen et al,1958). These authors state that the essential mechanism in the trapping and stabilisation of unpaired electrons is the existence of ring clusters, which possess a high degree of resonance energy available for stabilisation of electrons. They further state that the probable origin of the radicals is in bond breakage around

the edge of the carbon clusters, and in the formation of defects in the ring packing, five and seven-membered rings producing internal trivalent carbon atoms.

The paramagnetic resonance absorption of side-stream smoke tar was measured. It contained a free radical concentration of 50%, approximately, that of main-stream smoke tar. This refers only to radicals of the stable group.

Section C (ii). Experiments on smoke solutions using the stable free radical α α'-Diphenyl-β-Pycryl Hydrazyl (D.P.P.H.)

D.P.P.H. dissolves in organic solvents to give a violet coloured solution. It was found that such solutions could be decolourised by cigarette smoke, or by freshly prepared benzene solutions of cigarette smoke. The progress of the reaction could be followed spectroscopically by observing the decrease in absorption at the 520 mμ maximum of D.P.P.H. The technique employed for such measurements has been described in the "Methods" portion of the present thesis.

The presence of reducing substances including free radicals was indicated. Approximately 60% of the reactive material was water soluble, reflecting the presence of considerable quantities of polar reducing substances in cigarette smoke. Such water soluble material was D.P.P.H. - reactive but not light - sensitive, as irradiation with an open-arc mercury vapour lamp at 15 cms for 3 hours did not appear to affect the reaction rate with D.P.P.H. The smoke from one cigarette (smoked into a litre of benzene) had a reducing power approximately equal to a $0 \cdot 5 . 10^{-4}$ molar solution of Hydroquinone.

The non-polar D.P.P.H. reactive material was found to be

light sensitive, an irreversible decrease in activity being found on exposure to daylight. This effect was more pronounced on exposure to U.V. light.

The light-sensitive material seemed to be a product of the combustion of the cigarette, as previous extraction of the tobacco with acid, alkali and/or organic solvents did not give rise to a light-insensitive smoke. The diminution of activity occurred under oxygen and nitrogen atmospheres to an equal extent, so that atmospheric oxygen did not seem to be involved. A parallel was noted with the work of Druckery and Schmähl (1955) and Johnston (1957), who showed that the fluorescence intensity of smoke solutions decreased on light exposure. These workers were unable to explain the nature of the labile components. In the present instance, it was decided to test whether the decrease in D.P.P.H. activity and the decrease in fluorescence intensity were related phenomena, and if such a prediction proved correct, to attempt an explanation.

In order to circumvent the large background D.P.P.H. activity of the polar reducing substances present in the unsmoked tobacco and distilled over in the smoke, cigarettes were extracted with water (by refluxing for 2 hours) before

smoking and then dried to their normal moisture level of 10 - 12%. The smoke from one such cigarette in about 250 ml of benzene afforded a convenient solution for both fluorescence and D.P.P.H. activity measurements. The solution was irradiated in tubes for 24 hours, tubes being withdrawn every hour for the first seven hours and at three hourly intervals thereafter. The % age decrease in fluorescence and D.P.P.H. activity was correlated with concentration by reference to the appropriate dilution curves (Figs. 13a and b). The average result of three such sets of measurements are shown in Figure 14. A remarkably close correspondence was found.

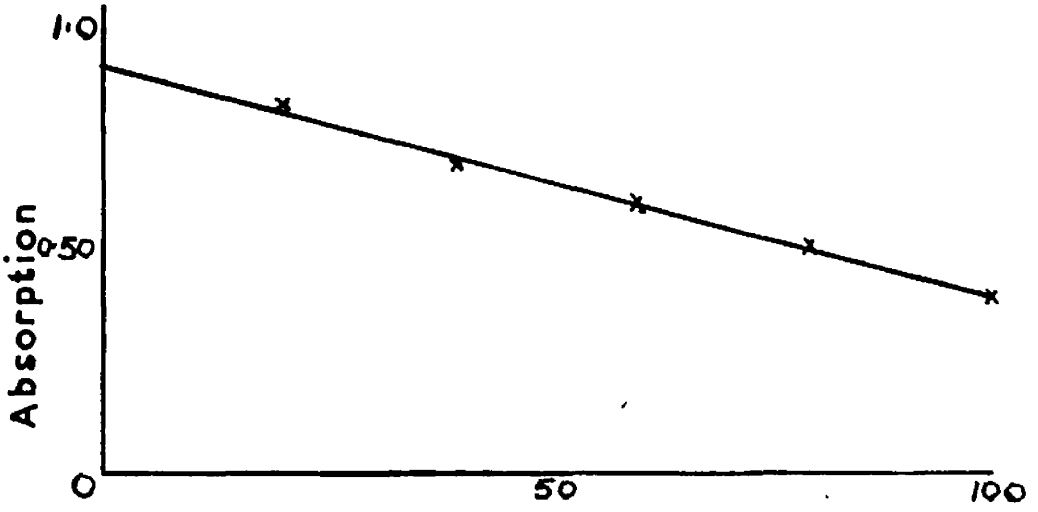
An analysis of the curves shows them to be discontinuous at 2 points, the 4 and 6 hour irradiation points. A decrease of about 60% in the concentration of labile components occurs in 24 hours, the major portion (50%) occurring in the first 7 hours of irradiation. The reaction had 1st order kinetics, as a straight line was obtained when $\ln \frac{a}{a-x}$ was plotted against t. This is shown for the reaction over the first 4 hours (Fig. 15). A mean rate constant of 0·015, min^{-1}, was obtained for the fluorescence and D.P.P.H. light reactions. The three different rate constants reveal the presence of three different components.

The free radical concentration of a smoke solution as

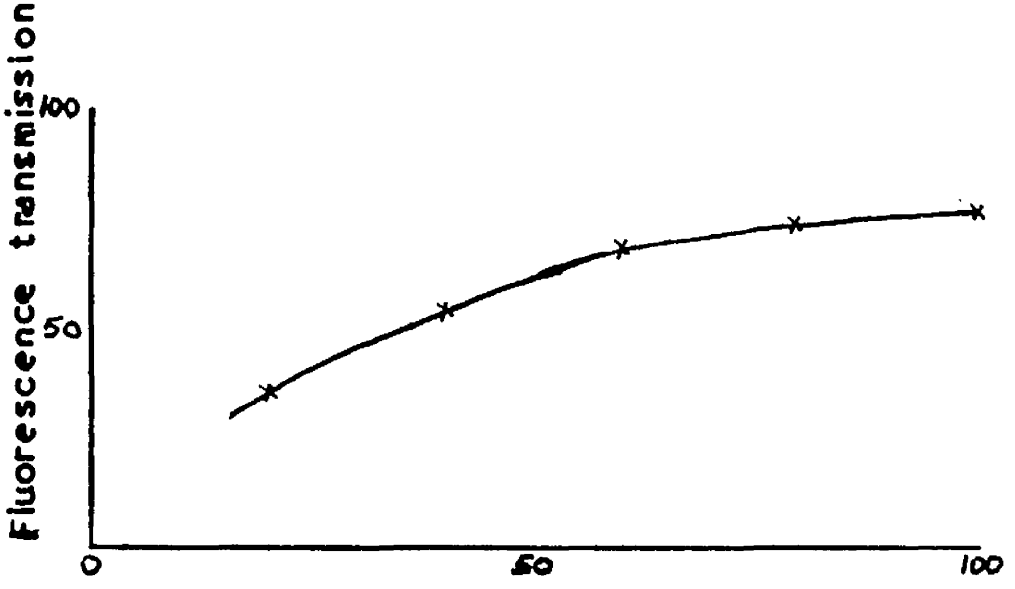
measured by the electron resonance spectrometer following 6 hours irradiation under the same conditions showed a 40% decrease approximately. This corresponds extremely well with the fluorescence and D.P.P.H. activity decrease.

The light-sensitive components of cigarette smoke appear therefore to be free radicals of the stable group (mentioned in the previous section), a light titration of which revealed the presence of at least three species of different stability.

Benzene extracts of atmospheric soot reacted with D.P.P.H. On a dry weight basis, the cigarette smoke proved 9 times more active than such extracts. They were not light sensitive.

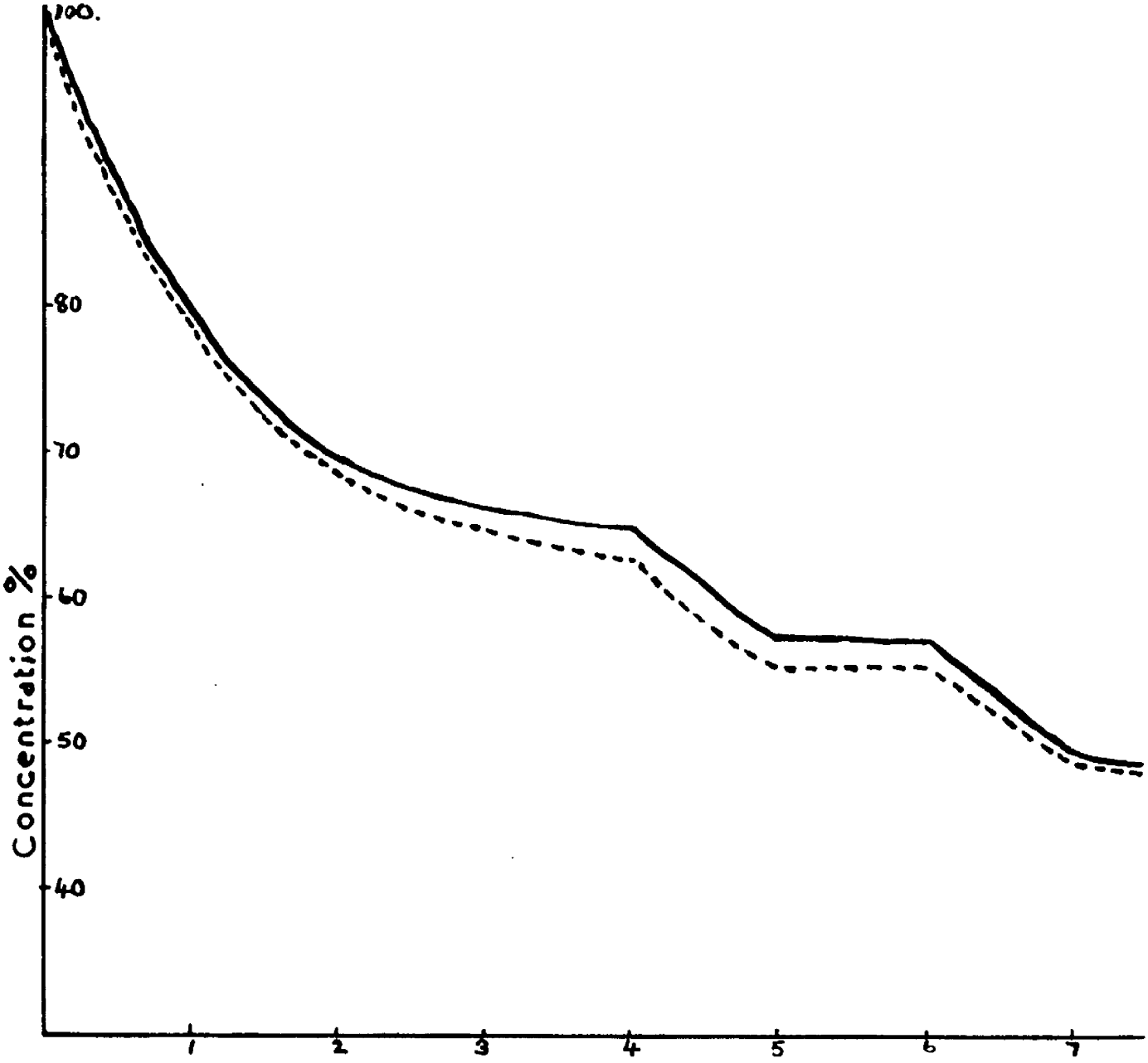


(a) DPPH Absorption against concentration of Smoke solution



(b) Fluorescence against concentration of Smoke solution

FIG. 13.



Light dependence of Cigarette Smoke solutions.

——— DPPH Reaction

------ Fluorescence

FIG. 14.

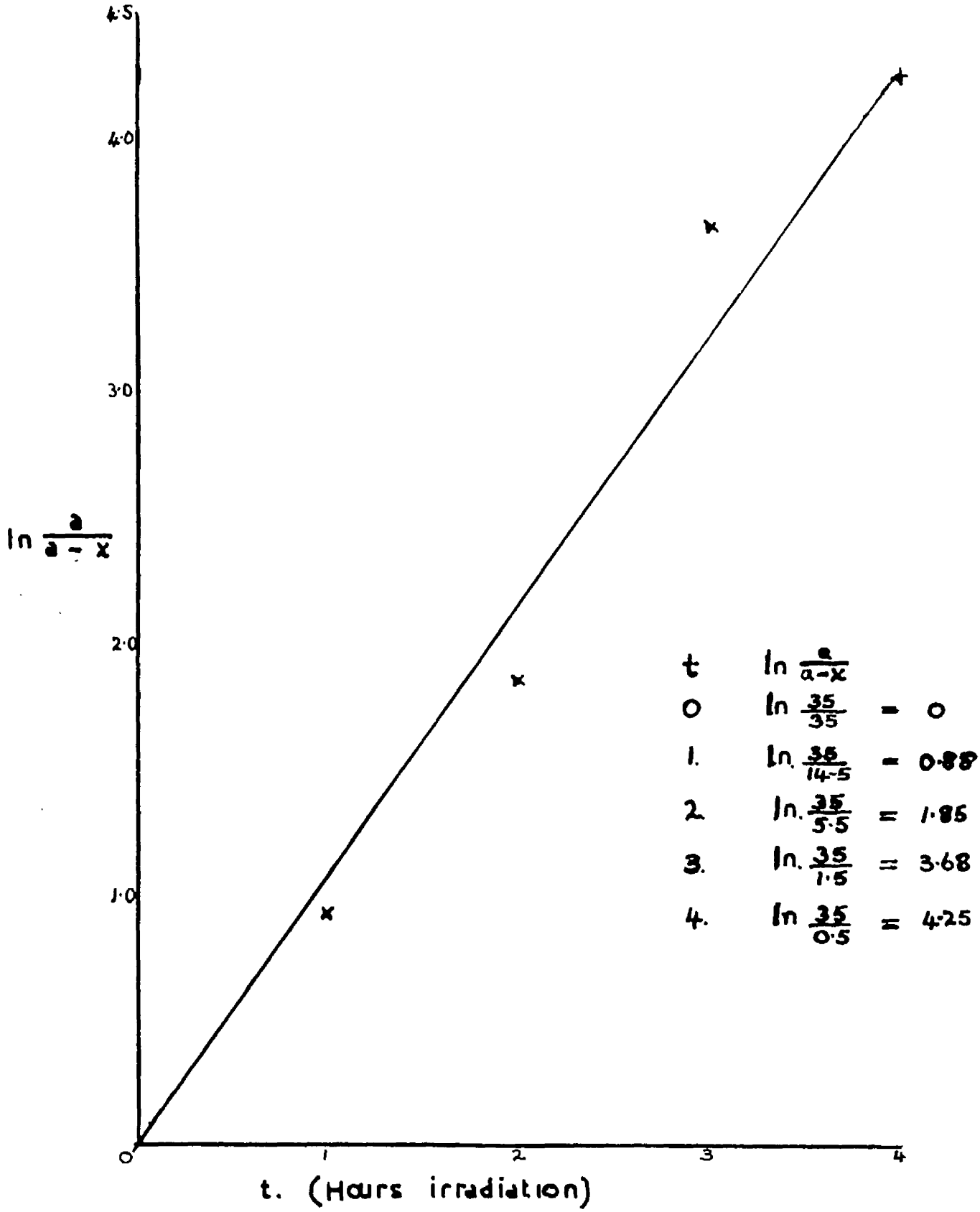


FIG 15. ORDER OF REACTION for initial 4 hrs irrad.

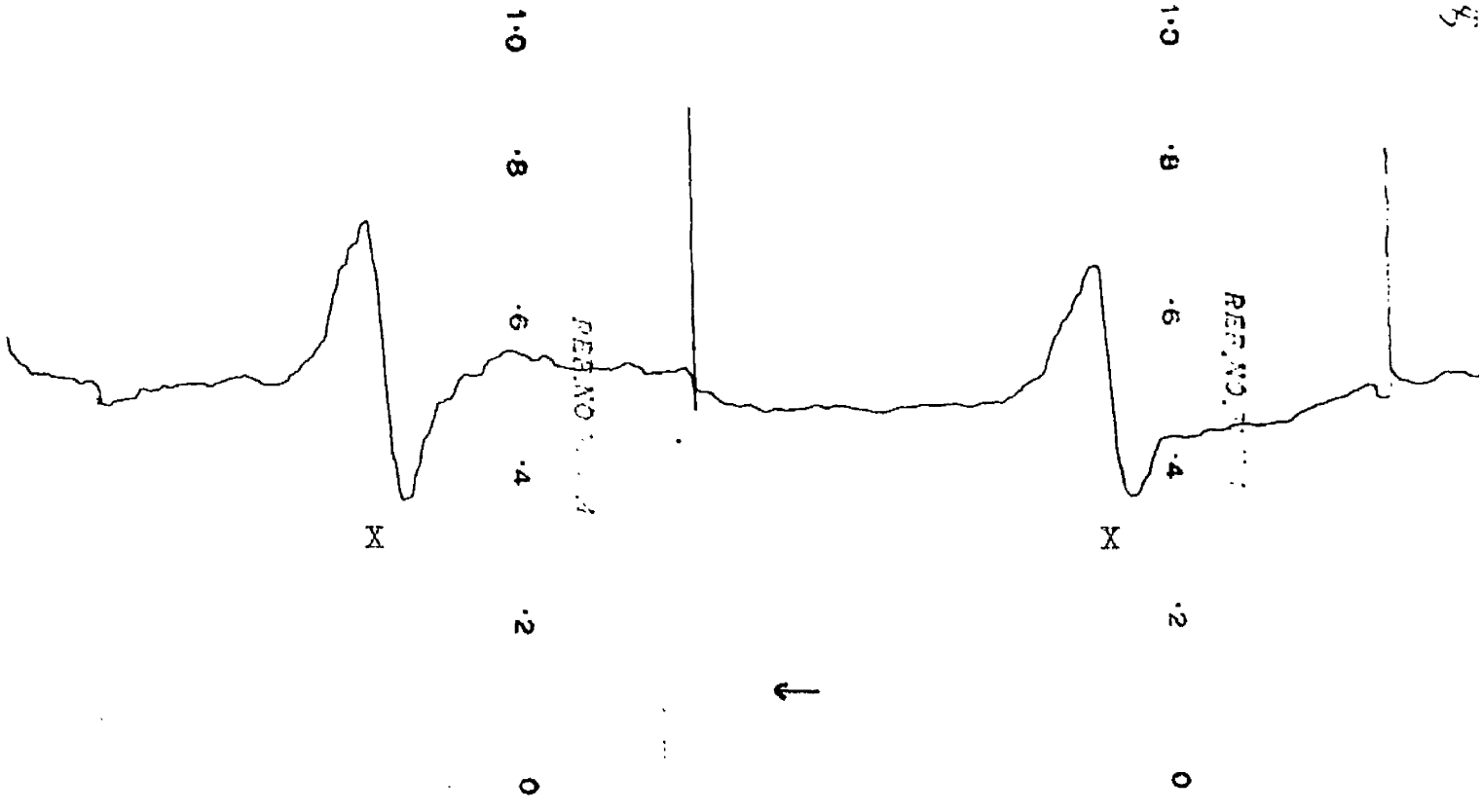


FIG. 16. Free radical signal given by cigarette smoke condensate. The signal (derivative) is denoted by X.

DISCUSSION

Particulate Air Pollutants

In the breakdown of the so-called 'Urban factor', in human bronchogenic carcinoma, a key role for the aromatic hydrocarbon component was postulated (see 'Introduction). For this, evidence was brought forward, (i) from the epidemiology of tar-fume cancer, (ii) from the fact that bronchogenic carcinoma can be reproduced in rodents with members of this class of compound (Andervont, 1939), (iii) from the fact that among such members a versatility to evoke tumours in different tissues and various species exists, (iv) from the fact that a tissue response (a hyperplastic reaction) was obtained from human foetal lung tissue in organ culture following application of 3,4-Benzopyrene (Lasnitzki,1958) while no such response was obtained when the non-carcinogenic compound pyrene was applied (idem, personal communication).

In order to provide a rational basis for this hypothesis it became necessary, firstly, to determine whether, in urban atmospheres, a variety of hydrocarbons, carcinogenic under laboratory conditions, existed. The proposition is made that the greater the number of different carcinogenic hydrocarbons present, the greater the probability that one of them is car-

cinogenic for human lung tissue. It is assumed that pure compounds which are inactive under laboratory conditions are also inactive with respect to humans.

The presence and intensity of absorption maxima combined with fluorescence spectral analysis were the only means employed to identify the aromatic hydrocarbons separated from the various soots. The latter method proved invaluable in segregating the various compounds, especially those which were eluted subsequent to 3,4-Benzopyrene from the chromatographic column. Nevertheless, compounds were detected in this range which did not give striking fluorescence spectra under the conditions used. In such cases, U.V. absorption analysis was employed.

In many instances superimposition rendered the interpretation of both absorption and fluorescence spectra difficult. Further, the quantities involved, especially of some compounds eluted subsequently to 3,4-Benzopyrene (if this compound is taken as a marker) were in the microgram range and frequently the absorption intensities of maxima were well below 0.1, i.e. in the inaccurate range where errors of up to 200% can result. Nevertheless, the methods adopted suggest that for a fuller analysis of soots, larger quantities of starting material should be worked up,

alternate adsorption chromatography on Alumina and Silica Gel employed, followed throughout by segregation on the basis of fluorescence and absorption analysis until finally an adequate paper chromatographic technique - such as that of Tarbell et al (1955) - be used to achieve separation of closely related compounds.

Compounds ranging from the relatively simple Naphthalene containing two fused benzene nuclei to Coronene, and what is tentatively identified as 1,12,2,3-Dibenzoperylene, which contain seven fused benzene nuclei, were encountered in the analyses of the various soots. In general, formation of the simpler compounds containing from two to five rings, seemed to be favoured in the combustion processes involving Diesel and Petroleum fuels.

Individual carcinogenic compounds.(a) Qualitative.

The Atmospheric Soot was found to contain, in addition to the traditional carcinogen 3,4-Benzopyrene, a compound which is tentatively identified from fluorescence and absorption data, as 3,4-Benzofluoranthene. This compound has been reported by Kotin (1958)* as being present in a sample of atmospheric soot, and by Wynder* (1958) as being present in

* Personal communication

cigarette smoke tar. These workers reported the compound to be strongly carcinogenic for mice, in contrast to 11,12-Benzofluoranthene which they reported to be inactive. The latter compound, which has been found in all the soots examined and in cigarette smoke tar, can easily be identified in chromatographic fractions by its striking fluorescence spectrum - which, incidentally, is not unlike that of 3,4-Benzopyrene. 3,4-Benzofluoranthene was also detected in Petrol and Diesel Exhaust soots.

Potent carcinogens of the Dibenzopyrene series were detected in Petrol Exhaust Soot. These included the 1,2,3,4- and 1,2,4,5-derivatives with possibly trace amounts of the 3,4,8,9-compound. 1,2,4,5-Dibenzopyrene has been reported by Arbuzov and Grechkin, (1952) as being highly carcinogenic for rodents. This fact is contrary to the Pullman theory, which would designate a low K region value - and consequently low activity, if any - to this compound (Pullman and Pullman, 1955). 1,2,3,4-Dibenzopyrene has also been detected in the Diesel Exhaust Soot. This compound, which is associated with a green fluorescence was preceded chromatographically by a blue-green fluorescent unidentified component in the exhaust soots. These compounds and the 1,2,4,5-Dibenzopyrene were not detected in the general atmospheric soot sample, suggest-

ing that no other prominent source of atmospheric pollution with these compounds existed or that they were readily destroyed in the atmosphere. Two further compounds were isolated from the Petrol Soot sample (Fractions L2 and L3), which resembled spectrographically compounds isolated from horizontal retort tar by Berenblum and Schoental (1947). These workers showed that two fractions which recorded main fluorescence bands at 391 mu and 385 mμ were carcinogenic for rabbit skin.

(b) Quantitative

Quantitative differences occurred in the relative concentrations of the hydrocarbons among the different soots. The demonstration of 3,4-Benzopyrene as the hydrocarbon of highest concentration of those estimated in the petrol soot sample is at variance with the data of Kotin et al (1954) which would place Pyrene at higher concentration to the benzopyrene. However, Kotin* (1958) now agrees with the benzopyrene order of concentration for the three soots, as shown by Lyons and Johnston (1957), i.e. - Petrol Soot > Atmospheric Soot > Diesel Soot. The low concentration of 3,4-Benzopyrene in the Diesel Soot conforms to the levels achieved by Commins, Waller and Lawther (1956).

* Personal communication

On a free carbon basis, Petrol Soot is shown to contain 3,4-Benzopyrene in a concentration four times greater than that of general atmospheric soot. On a whole soot basis, the concentration is approximately three times greater. Therefore, it would seem that Petrol Exhaust soot is a notable source of atmospheric enrichment with regard to 3,4-Benzopyrene. A similar situation holds for the carcinogenic Dibenzopyrenes which were detected in vehicular exhaust soot but which were below the limit of detection in the atmospheric soot due either to dilution by admixture with general soot particles, to destruction in the atmosphere, or both.

The presence of considerable quantities of associated oil in the vehicular exhaust soots was noted. This fact may be of importance in the light of recent work by Horton et al (1957) who showed that for C3H mice, skin carcinogenesis could be accelerated by using aliphatic and related hydrocarbons as solvents for the carcinogenic agents 3-Methylcholanthrene or 3,4-Benzopyrene. These workers found that for n-paraffins, the accelerating activity rose sharply as the chain length increased from 8 to 12 Carbon atoms. The oils recovered in the present experiments, being, it is thought,

composed of unburnt fuel and lubricant, almost certainly contain n-paraffins of this order. The ratio of oil to 3,4-Benzopyrene in the case of the Petrol Soot was 100 to 0·16 g., or, put another way, the benzopyrene existed on carbon particles, in oily solution of concentration 0·16 per cent by weight. This fact is interesting in the light of Horton's work, who found a relative accelerating activity of 2·3 to 2·5 with an n-dodecane solution of benzopyrene of concentration range 0,20 to 0·04 per cent by weight. A relative accelerating activity of 2·3 means that tumours are produced 2·3 times as rapidly by a given solution as would be expected if the solvent had no accelerating capacity.

Conclusions.

A variety of aromatic hydrocarbon types, carcinogenic under laboratory conditions, have been found in particulate air pollutants. Results suggest that vehicular exhausts, notably the exhaust soots of automobiles using Petrol as fuel, are important sources, leading to the enrichment and enlargement of the atmospheric pool of carcinogens.

Kotin (1956) quotes data obtained from E.C. Hammond showing a parallelism between increased motor fuel consumption and the rise in lung cancer incidence and mentions that

such environmental factors are more capable of correlation with a proposed two decade latent period for the cancer. In the light of the present results, therefore, increased motor fuel consumption has probably resulted in a progressive enrichment of the atmosphere in carcinogenic hydrocarbons

Waller (1952) quoting many references, states that the exhaust particles of the internal combustion engine "may be quite near to the range which undergo maximum retention in the lung".

Falk et al (1958) in investigating the fate of retained soot particles, have shown that plasma proteins are capable of eluting aromatic hydrocarbons from the particulate structures in an order corresponding to the elution of such compounds from columns of alumina by non-polar solvents.

Peacock et al (1949) have postulated that prolonged contact of carcinogenic hydrocarbons with the tissues is an important parameter in the induction of malignancy. This principle has recently been shown to be valid for the production of bronchogenic carcinomas in rats using radio-active salts, by Camber and Watson (1958) who showed that duration of radiological insult was a prime

factor in determining the degree of hazard from inhaled radio-active particulates.

The following course of events, compatible with the chemical and biological findings and leading to the induction of lung tumours, can therefore be envisaged. Soot particles, containing a variety of agents carcinogenic under laboratory conditions, one or more of which may be carcinogenic for human respiratory epithelium, are deposited and retained in the respiratory tract where they may be phagocytosed. These particles then act as vectors to release and maintain a prolonged focal concentration of the carcinogen(s), titrating it against succeeding cell generations until a susceptible generation undergoes an initiating action through its agency.

The postulated "urban factor" can thus, by the consideration of exogenous parameters, be furnished with a rational basis. However, the importance of endogenous parameters whether systemic, hormonal or broadly, genetic, cannot be over-emphasised, especially when the extrinsic carcinogenic stimuli are weak. In the lung cancer aetiology being discussed in the present thesis, the extrinsic stimuli may well

fall into this category, as the following comparison shows. Approximately 90% of heavy cigarette smokers never develop lung cancer, the proportion of lung cancers being very much less in the general population of smokers and non-smokers alike. The carcinogenic stimuli affecting this general population then must be considered as far weaker than e.g. that stimulus (radioactive dust) which, in the miners of Schneeberg was responsible for a death rate from cancer of the lung of 75% of all deaths (Hueper, 1954).

The "urban factor", as the most recent statistical analyses have emphasised (Stocks, 1957, Hammond and Horn, 1958), diminishes in an inverse relationship with a postulated cigarette smoking factor. For the cigarette smoker, a special potentially carcinogenic environment exists. The answers to whether this postulated special environment could be explained in terms of concentrations of carcinogenic aromatic hydrocarbons or other theoretically possible carcinogens to which non-smokers, urban and rural-living alike, are not exposed, were sought in the second portion of the present thesis. But before discussing these results, the question of whether the role of cigarette smoke in the pathogenesis of lung cancer is merely that of a co-factor, must be

considered. It has been suggested that the nitrogenous bases (e.g. Pyridine) of cigarette smoke act as eluents for the aromatic hydrocarbons of atmospheric soots deposited in the respiratory tract, thereby facilitating carcinogenic action (Cooper, 1954). It could also be suggested that cigarette smoke has a general or particular systemic action which results in the creation of an environment in the tissues of the respiratory tract favourable to carcinogenesis by the carcinogenic hydrocarbons of deposited atmospheric soot. In both of these cases, as atmospheric soot is the limiting factor, a pronounced urban-rural ration could be expected for cigarette smokers. As this is contrary to the facts, the co-factor hypothesis as stated is unlikely to be important, and the rationale must be orientated to the consideration of cigarette smoke as being carcinogenic per se.

Cigarette Smoke. Aromatic Hydrocarbons.

The analytical methods used were the same as those used for the soots. Considerable more difficulty however was encountered in the purification of compounds from cigarette smoke tar than from the soots. Nevertheless, a number of compounds were identified with reasonable certainty. 3,4-Benzopyrene was detected and further carcinogens were detecte

in fractions which preceded and succeeded this compound on the chromatogram. Among the former were the weakly carcinogenic 3,4-Benzophenanthrene, 1,2-Benzanthracene and 1,2-Benzopyrene. Also occurring in this chromatographic region were benzanthracene derivatives tentatively identified as 5,6 and 6,7-Cyclopentano - 1,2-Benzanthracene. These compounds have been shown to be carcinogenic for mice (Cook, 1931; Barry et al, 1935; as quoted by Hartwell, 1951). In the present instance, as reference compounds were not available for quantitation, the approximate concentration levels for both was gauged by comparison with a 1,2-Benzanthracene standard. A concentration level not exceeding 1 µg per 100 cigarettes smoked for each of the compounds was obtained.

The following carcinogens were detected in the fractions eluted subsequently to the 3,4-Benzopyrene; 1,12-Benzoperylene (which is reported by Cooper and Lindsay, 1955, as having slight activity for mice) at a concentration level of approximately 2 µg per 100 cigarettes smoked; 1,2,3,4-Dibenzopyrene (which Bachmann et al, 1937, and Cook and Kennaway, 1938, describe as a carcinogenic agent of considerable potency on the basis of the mouse skin test) at a concentration of

1.6 µg per 100 cigarettes smoked (Lyons, 1958); 3,4, 8,9-Dibenzopyrene in trace amounts. Also 1,2,7,8-Dibenzofluorene was tentatively identified and absorption peaks suggesting the presence of other members of this group obtained. The former compound was found to be carcinogenic for the skin of mice by Badger et al (1940), but some doubt then existed as to the structure of the substance used. More recently, Flegal et al (1951) showed the compound to be non-carcinogenic for mouse skin. Rosenblum and Schoental (1947) reported obtaining two fractions from horizontal retort tar having fluorescence maxima at 412 mµ and at 385 mµ, which were active for mouse and rabbit skin. Both of these fractions occurred after the Benzopyrene fraction chromatographically. In the present work fractions were obtained (H3 and H1) which may contain the same carcinogenic agents, as similar fluorescence maxima were shown. Recently, Schoental (1957) has reported the isolation of the potent carcinogen 3,4,9,10-Dibenzopyrene from the horizontal retort tar. This compound has not been encountered in the present investigations. Bonnet and Noukoma (1956) in an investigat-

lon of cigarette smoke tar have stated that, apart from the detection of a compound which they designated as 3,4,9,10-Dibenzopyrene, no further compounds could be detected in the chromatographic fractions succeeding 3,4-Benzopyrene. This is in marked contrast to the observations of the present analysis, where, at least eighteen compounds, nine of which remain unidentified, were encountered. It is thought that the Swiss workers are in error in designating the compound eluted subsequently to 3,4-Benzopyrene, 3,4,9,10-Dibenzopyrene. A compound was detected in the present analysis (Fraction B5) which succeeded 3,4-Benzopyrene on the chromatographic column and which had peaks in the visible end of the spectrum, at 435, 396 and 377 mμ similar to those of the dibenzopyrene. The fluorescence spectrum of the compound however was completely different from that of an authentic specimen of 3,4,9,10-Dibenzopyrene. Furthermore the latter compound's chromatographic behaviour (it is the most strongly adsorbed of the dibenzopyrenes) was incompatible with its elution in the fraction (B5) indicated.

Wynder and Wright (1957) observed that a Hexane fraction, low in 3,4-Benzopyrene was more strongly carcinogenic for

rabbit skin than a succeeding Carbontetrachloride fraction which contained the bulk of the benzopyrene. This bore a superficial similarity to Berenblum and Schoental's (1947) finding with respect to a coal tar fraction. It is not known whether any of the carcinogens found preceding 3,4-Benzopyrene, in the present investigation are active for rabbit skin.

Aromatic hydrocarbons. Quantitative relationships

The quantity of 3,4-Benzopyrene recovered in the present analysis corresponds with that obtained by Cooper and Lindsay (1955), Bonnet and Neukomm (1950) and Laterjet et al (1956).

Wynder and Wright (1957) estimated the benzopyrene concentration of tar from American cigarettes at less than 2 p.p.m. whole tar. They stated that no parity existed between Benzopyrene content and biological activity e.g. for mouse skin the $C.Cl_4$ fraction (containing the bulk of the Benzopyrene at an approximate concentration of 0·0002g%) had an activity corresponding to a benzopyrene solution of concentration 0·005 g%. The cigarette tar was potentiated 25-fold. It was stated (loc.cit) that spectrographic evidence indicated the presence of compounds in concentrations 50 times that of the Benzopyrene. The author does not

understand how such an observation on unknown compounds can be adduced from spectrographic data, as considerable variation of intensity is known to exist even among isomeric hydrocarbons (Schoental and Scott, 1949). Evidence from the present work suggests that the biological activity of cigarette tar for the skin of rodents is largely due to the summative action of low levels of various carcinogenic hydrocarbons. Passey (1957) in an evaluation of the results of his own experiments and those reported from other laboratories in Great Britain, gave the generally accepted opinion when he stated "... it is clear that the carcinogenic action of cigarette tar on the skin of mice is a weak one".

In considering quantitative relationships between cigarette smoking and general atmospheric pollution, the following points can be made. If 2 µg of 3,4-Benzopyrene are inspired and retained by the smoker for every 100 cigarettes smoked, then if he is a "heavy smoker" consuming 30 cigarettes per day, an annual exposure of about 200 µg of Benzopyrene results from this source. An average non-smoker, as calculated from Waller's data (1952), inspires

approximately the same quantity of Benzopyrene per annum from the atmosphere. The quantity is subject in this case, however, to devaluation due to the fact that the percentage of general atmospheric particulate pollutants of a size greater than 2·5 μ will be largely arrested by the efficient nasal filter (Blacklock et al, 1954). An assumed 25-fold potentiating factor for cigarette smoke could conceivably be counterbalanced by a corresponding potentiation with respect to atmospheric pollutants. The average urban-dwelling cigarette smoker (30 cigarettes per day) has an exposure to benzopyrene approximately twice that of his non-smoking neighbour. This ratio of two to one will diminish with increasing urbanisation. But while the consideration of Benzopyrene concentrations may not harmonize with the statistical findings, that for other carcinogenic hydrocarbons may. One such hydrocarbon 1,2,3,4-Dibenzopyrene, found at a concentration if 1·6 μg per 100 cigarettes smoked, was not detected in a sample of general atmospheric soot. In this case then, the cigarette smoker has an exposure to a specific carcinogen at a concentration not realised by his non-smoking neighbour, irrespective of the degree of urbanisation.

Reality is withheld from these considerations by virtue of the fact that knowledge of the tolerance level for man is not known. For mouse skin, Poel (1956) showed that as little as 120 µg of 3,4-Benzopyrene applied by the drop method over a period of 10 months produced 20% of tumours. A corresponding period for man would be 20 years, which may be regarded as being equivalent to the latent period for lung cancer. A cigarette smoker smoking 30 cigarettes per day could receive an exposure of 4000 µg Benzopyrene in that period. In this case, the area exposed to the benzopyrene is very much greater than in the mouse experiment. However, local concentrations are likely to occur in areas where the ciliated epithelium has desquamated through the action of inspired non-specific irritants.

In the absence of knowledge of the tolerance level, any exposure to carcinogenic hydrocarbons must be regarded as synonomous with an adverse effect.

Whether any significance attaches to the form in which the hydrocarbons of cigarette smoke are presented to the respiratory epithelium is at present unknown. Quantities of plant steroidal material have been detected in cigarette smoke (Wynder and Wright,1957) which may act as carriers for

the ingress of the hydrocarbons into the tissues. But also removal of the hydrocarbons from foci of action in the cells could conceivably be facilitated by the same mechanism. In this regard, it is interesting to recall the work of Jaffe and Eliassow (1927) who, in confirming earlier work by Twort and Twort, showed that the carcinogenic potency of tar was decreased by the addition of cholesterol to the tar.

It was considered that the postulated special environment of the cigarette Smoker could not be satisfactorily explained in terms of carcinogenic hydrocarbons alone and thus it seemed of interest to examine cigarette smoke for the presence of other materials of possible carcinogenic significance, especially, if such were unique to cigarette smoke. S uch an investigation led to the demonstration of cigarette smoke as a reducing agent and to the detection of free radicals.

The reducing properties of cigarette smoke.

As mentioned in the 'Results' section, the smoke from one cigarette had a reducing or anti-oxidant capacity in Benzene equivalent to a $0{\cdot}5 \times 10^{-4}$ molar solution of hydro-

quinone, as indicated by use of the reagent $\alpha\alpha'$-Diphenyl-β-Picrylhydrazyl. The effect of an intermittant reducing environment on the tissues of the respiratory tract is unknown. One is tempted to think along the lines of the Warburg theory (reviewed by Warburg, 1956), and to refer to the induction of malignancy by intermittant anaerobiosis (Goldblatt and Cameron, 1953), and to Warburg's statement that malignancy 'caused' by certain thiols (reducing agents) had its origin in a biochemical lesion at the cell respiration level. The Warburg theory cannot be said to have been proved, however (see Weinhouse, 1956).

On another line of speculation, the possibility of anti-oxidants stabilising a relatively labile carcinogen in situ cannot be precluded (Chevallier et al., 1946).

The free radicals of cigarette smoke.

Two broad classes of free radicals were detected and estimated in cigarette smoke condensates.

Class I: An unstable, highly reactive class which could only be demonstrated in condensates obtained by refrigerating fresh cigarette smoke to liquid oxygen temperatures.

Class II: A comparatively stable class, which represented the radicals that remained following warming of the refrigerated condensate. The latter amounted to approximately 17% of the total free radicals.

On a weight basis, side-stream smoke was found to posses about half the concentration of Class II radicals as main-stream smoke.

Class II radicals were found to consist, on a chemical basis, of alkaline, acid and neutral species. Over 80% of the radicals could be desorbed from Alumina by solvents of increasing polarity. The radical molecules were fluorescent, which fluorescence, on exposure to light, was found to decrease discontinuously, indicating the presence of at least three species of different stability. The reaction of the free radicals with light followed first order kinetics

A non-smoker in a smoking compartment can be expected to have an exposure to comparatively low levels of the more stable of Class II radicals from pollution of the ambient air by side-stream smoke.

A benzene extract of general atmospheric soot had a

free radical activity of 100 x 10^{15} free electrons per g. This extract amounted to about 25% of the total soot by weight. On the basis of Waller's figures (1952), 1·43 g of soot is inspired by the 'standard man' per annum. If no arrest of the soot particles occurs and the total amount inspired is retained, an exposure to approximately 36×10^{15} free electrons occurs. A 'heavy' smoker who consumes 30 cigarettes per day has a yearly exposure of about 330 x 10^{15} free electrons, i.e. nearly ten times the quantity received by the non-smoker. The radicals from the general atmosphere soot, in contrast to those from cigarette smoke, are not light sensitive, i.e. are of a higher order of stability.

It can be stated then, that exposure to Class I and Class II free radicals represents a feature which is special to the cigarette smoker. Their mode of action in the cell could conceivably involve a distortion of macromolecules through translocation of electrons, or a deletive action through interaction with cellular free radicals.

Most bioassays in the field of tobacco carcinogenesis involve the use of smoke condensates taken up in organic solvents, or tars produced by trapping smoke in such solvents. It is now clear that such materials are deficient in the highly reactive Class I free radicals.

SUMMARY

1. From a background of epidemiological, statistical, clinical and biological information, evidence has been adduced for the consideration of carcinogenic aromatic polycyclic hydrocarbons as occupying a key role in the pathogenesis of the human non-adenomatous lung cancers which are relatable to general atmospheric pollution.

2. Assays of particulate air pollutants for the hydrocarbons which are carcinogenic under laboratory conditions have been made, using the methods of repetitative adsorption chromatography, U.V. absorption spectrophotometry and fluorescence spectrography. The pollutants investigated were samples of Petrol and Diesel engine exhaust soots, and a sample of general atmospheric soot. The relative importance of the former two soots as sources of atmospheric enrichment in carcinogenic hydrocarbons was gauged by comparison with the latter soot.

3. Petrol Exhaust Soot was found to be an important source of atmospheric enrichment in 3,4-Benzopyrene. The following carcinogens have also been identified in this soot:

1,2,3,4-Dibenzopyrene, 1,2,4,5-Dibenzopyrene and 3,4-Benzofluoranthene.

4. The Diesel Exhaust Soot was not found to be a good source of 3,4-Benzopyrene, in contrast with the Petrol Soot. However, like the latter, it was found to be a source of 1,2,3,4-Dibenzopyrene and 3,4-Benzofluoranthene. Trace quantities of this latter compound, whose strong carcinogenicity for mice has only recently been discovered, have been detected in the sample of general atmospheric soot, and may have had its origin in vehicular exhausts.

5. The biological significance of an associated oil in the case of the exhaust soots as a possible adjuvant factor was discussed.

6. The fluorescence and absorption features of many compounds, not hitherto recorded in the present connection, have been presented.

7. Evidence suggesting that side-stream and exhaled cigarette smoke contribute to the general atmospheric pool of 3,4-Benzopyrene has been obtained.

8. Accepting the validity of the statistical findings which - for men - causally relate cigarette smoking with non-adenomatous lung cancer, a special carcinogenic environment for cigarette smokers was postulated. Cigarette smoke was considered as being carcinogenic per se. A variety of carcinogenic hydrocarbons were demonstrated in cigarette smoke in quantities less than 2 p.p.m. of whole tar, for those estimated. The carcinogenic hydrocarbons found were, 3,4-Benzopyrene and 1,2,3,4-Dibenzopyrene as well as the following compounds for which some carcinogenic activity under laboratory conditions has been demonstrated. 1,2-Benzanthracene, two 1,2-Benzanthracene derivatives, 3,4-Benzophenanthrene, 1,2-Benzopyrene, 1,12-Benzoperylene. Attention was further drawn to three other fractions (one of which contains what is tentatively identified as 1,2,7,8-Dibenzofluorene) which may be carcinogenic.

9. The cigarette smoker is shown to have an exposure to 1,2,3,4-Dibenzopyrene at a concentration (1·6 µg per 100 cigarettes smoked) which is not realised by his non-smoking neighbour.

10. Doubt remained as to whether the proposed special environment was explicable solely on the basis of the traditional carcinogenic hydrocarbons found, and consequently cigarette smoke was investigated for the presence of other factors of possible carcinogenic significance. Such investigations led to the estimation of the reducing or anti-oxidant activity of cigarette smoke by use of the stable free radical $\alpha\alpha'$-Diphenyl-β-Picrylhydrazyl, and to the detection and estimation of free radicals in cigarette smoke by the Electron Paramagnetic Resonance Method.

11. The smoke from one cigarette in Benzene was found to have a reducing power equivalent to an $0{\cdot}5 \times 10^{-4}$M solution of Hydroquinone.

12. An estimated radical concentration of 6×10^{15} free electrons per g. tar was obtained from a cigarette smoke condensate which was trapped at liquid oxygen temperatures. On warming the condensate, a reduction of the radical concentration by a factor of six approximately, was observed, demonstrating the presence of a considerable concentration of very active radicals which recombined on warming the refrigerated condensate of fresh smoke.

The radicals that remained after warming the condensate were found to be comparatively stable, may be acid, alkaline and neutral; may be desorbed from Alumina with solvents of increasing polarity; are light-sensitive and have a fluorescence which, on exposure to light, decreases discontinuously, revealing the presence of species of varying stability.

BIBLIOGRAPHY

1. Andervont, H.B., Public Health Reps., U.S.A., 54, 1512, (1939).

2. Arbuzov, B.A., and Grechkin, N.P., Zhur, Obshchei Khim., 22, 1692, (1952). and Am. Chem. Abs., 47, 9953 h. (1953).

3. Arnstein, A., Wien. Klin. Wchnschr., 26, 748, (1913).

4. Bachmann, W.E., Cook, J.W., Dansi, A., de Worms, C.G.M., Hewett, L.C., and Robinson, A.M., Proc. Roy. Soc., B, 123, 350, (1937).

5. Badger, G.M., Cook, J.W., Hewett, C.L., Kennaway, E.L., Kennaway, N.M., Martin, R.H., and Robinson, A.M., Proc. Roy. Soc. B, 129, 439, (1940).

6. Berenblum, I., and Schoental, R., Bri. J. Exptl. Path., 24, 232, (1943).

7. Berenblum, I., Nature, 156, 601, (1945).

8. Idem and Schoental, R., J. Chem. Soc., 1017, (1946).

9. Idem, Bri. J. Cancer, 1, 157, (1947).

10. Blacklock, J.W.S., Kennaway, E.L., Lewis, G.M., and Urquhart, M.E., Bri. J. Cancer, 8, 40, (1954).

11. Blois, M.S., Biochim. Biophys. Acta, 18, 165, (1955).

12. Idem, Nature, 181, 1199, (1958).

13. Bonnet, J., and Neukomm, S., Helv. Chim. Acta., 39, 1724, (1956).

14. Brande, E.A., Brook, A.G., and Linstead, R.P., J. Chem. Soc., 3574, (1954).

15. Brocklehurst, B., J. Chem. Soc., 3318, (1953).

16. Bruos, A.M., and Barron, E.S.G., Ann. Rev. Biochem., 20, 350, (1951).

17. Camber, H., and Watson, J.A., A.M.A. Arch. Indust. Health, 17, 230, (1958).

18. Chevallier, A., Manuel, S., and Denoix P., Bull du Cancer, 33, 223, (1946).

19. Clar, E., "Aromatische Kohlenwasserstaffe", (Springer, Berlin, 1952).

20. Clemo, G.R., Chem. and Ind. (Rev.), 957 (1953).

21. Commins, B.T., Cooper, R.L., and Lindsey, A.J., Bri. J. Cancer, 8, 296 (1954).

22. Commins, B.T., Waller, R.E., and Lawther, P.J. Bri. Med. J., II, 753, (1956).

23. Cook, J.W. Hewett, C.L. and Hieger, I., J. Chem. Soc., 395, (1933).

24. Cook, J.W., and Kennaway, E.L., Amer. J. Cancer, 33, 53, (1938).

25. Cook, J.W., Schoental, R., and Scott, E.J.Y., Proc. Roy. Phys. Soc., 63, 592, (1950).

26. Cooper, R.L., Analyst, 79, 573, (1954).

27. Idem, Lindsey, A.J., and Waller, R.E., Chem. and Ind., 1418, (1954).

28. Cooper, R.L., and Lindsey, A.J., Bri. J. Cancer, 9, 304, (1955).

29. Doll, R., and Hill, A.B., Bri. Med. J. II, 739, (1950).

30. Iidem, Bri. Med. J. II, 1071, (1956).

31. Druckrey, H., and Schmähl, D., Science, 122, 421, (1955).

32. Ermala, P., and Holsti, L.R., Can. Res., 16, 490, (1956).

33. Falk, H. L., Markul, I., and Kotin, P., A.M.A. Arch. Ind. Health, 13, 13, (1956).

34. Falk, H.L., Miller, A., and Kotin, P., Science, 127, 474, (1958).

35. Gilbert, J.A.S., and Lindsey, A.J., Bri. J. Cancer, II, 398, (1957).

36. Goldblatt, H., and Cameron, G., J. Exptl. Med., 97, 525, (1953).

37. Goldschmidt, S., and Renn, K., Chem. Ber., 55, 628, (1922).

38. Haagen-Smit, A.J., Indust. and Eng. Chem., 44, 1342, (1952).

39. Haenszel, W., and Shimkin, M.B., J. Nat. Can. Inst., 16, 1417, (1956).

40. Hammond, E.C., Cancer, 7, 1100, (1954).

41. Idem and Horn, D., J. Am.Med. Assoc., 155, 1316, (1954).

42. Iidem, ibid, 166,1294, (1958).

43. Harlow, E.S., Science, 123, 226, (1956).

44. Härting, F.H., and Hesse, W., Vierteljahrsschr. f. Gerichtl. Med., 30, 296, (1879).

45. Hartwell, J.L., "Survey of compounds which have been tested for carcinogenic activity", Public Health Service, U.S.A., No.149, (1951).

46. Idem, Supplement I. (1957).

47. Hieger, I., Am. J. Cancer, 29, 709, (1937).

48. Horton, A.W., Denman, D.T., and Trosset, R.P., Can.Res., 17,758, (1957).

49. Hueper, W.C., R.I. Med.J., 36, 24, (1953).

50. Idem, "Recent developments in Environmental Cancer," Public Health Service, U.S.A., (1954).

51. Idem, Acta, U.I.C.C., 13, 97, (1957).

52. Ingram, D.J.E., Tapley, J.G., Jackson, R., Bond, R.L., and Murnaghan, A.R., Nature, 174, 797, (1954).

53. Ingram, D.J.E., and Tapley, J.G., Chem. and Ind., 568, (1955).

54. Ingram, D.J.E., "Spectroscopy at Radio and Microwave frequencies" (Butterworths, London, 1955).

55. Jaffe, R., and Eliasson, A., Verhandl. d. deutsch. path. Gesellsch., 22, 78, (1927).

56. Johnston, H., Nature, 180, 1350, (1957).

57. Kennaway, E.L., and Kennaway, N.M., Bri. J. Cancer, 1, 260, (1947).

58. Khanolkar, V.R., Can.Res., 4,313, (1944).

59. Kotin, P., Falk, H.L., and Thomas, M., A.M.A., Arch. Ind. Hyg. and Occup. Med., 9, 164, (1954).

60. Iidem, ibid., 11, 113, (1955).

61. Kotin, P., and Falk, H.L., Proc. 3rd Nat. Air Pollution Symposium, Calif. U.S.A. (1955).

62. Kotin, P., Can. Res., 16, 375, (1956).

63. Idem, Ibid, 18, 1, (1958).

64. Kreyberg, L., Bri. J. Cancer, 8, 599, (1954).

65. Kuratsune, I., and Hueper, W.C., J. Nat. Can. Inst., 20,37, (1958).

66. Lasnitzki, I., Proc. 7th Inter. Cancer Congress, 159, London, (1958).

67. Laterjet, R., Cusin, J-L., Hubert-Habert, M, Muel, B., and Royer, R., Bull du Cancer, 43, 180, (1956).

68. Levin, M.L., Goldstein, H., and Gerhardt, P.R., J. Am. Med. Assoc., 143, 536, (1950).

69. Liebow, A.A., "Atlas of Tumor Pathology", Sect. V., Armed Forces Inst. Path., Wash. D.C., U.S.A., (1952).

70. Lijinsky, W., Saffiotti, U., and Shubik, P., J. Nat. Can. Inst., 18, 687, (1957).

71. Lyons, M.J., B.E.C.C. Ann. Rep., 277, (1955).

72. Idem, Nature, 177, 630, (1956).

73. Idem and Johnston, H., Bri. J. Cancer, 11, 60, (1957a).

74. Iidem, ibid, 11, 554, (1957b).

75. Lyons, M.J., Gibson, J.F., and Ingram, D.J.E., Nature, 181, 1003, (1958).

76. Lyons, M.J., Nature, 182, 178, (1958).

77. Mayneord, W.V., and Roe, E.M.F., Proc. Roy. Soc., A, 152, 299, (1935).

78. Miller, J.A., and Bannmann, C.A., Can. Res., 3, 217, (1943).

79. Mills, C.A., and Porter, M.M., Can. Res., 17, 981, (1957).

80. Oppenheimer, B.S., Oppenheimer, E.T., Stout, A.P. Danishefsky, I., and Eirch, F.R., Science, 118, 783, (1953).

81. Iidem, Can. Res., 15, 333, (1955).

82. Passey, R.D., B.E.C.C. Ann. Rep., 65, (1957).

83. Pattle, R.E., Stretch, H., Burgess, F., Sinclair, K., and Edgington, J.A.G., Bri. J. Indust. Med. 14, 47, (1957).

84. Peacock, P.R., Beck, S., and Anderson, W., Bri. J. Cancer, 3, 296, (1949).

85. Pool, W.E., Science, 123, 588, (1956).

86. Idem and Kammer, A.G., J. Nat. Can. Inst., 18, 41(1957).

87. Poirier, R.H., Kahler, E.J., and Bennington, F., J. Org. Chem., 17, 1437, (1953).

88. Pullman, A., and Pullman, B., Adv. in Can. Res., 3, 117, (1955).

89. Riegel, B., Wartman, W.B., Hill, W.T., Reeb, B.B., Shubik, P., and Strangor, D.W. Can. Res., 11, 301, (1951).

90. Sadowsky, D.A., Gillian, A.G., and Cornfield, J., J. Nat. Can. Inst., 13, 1237, (1953).

91. Sanghvi, L.D., Rao, K.C.M., and Khanolkar, V.R., Bri. Med. J., I, 111, (1955).

92. Sannie, C., Biochem. J., 30, 704, (1936).

93. Schoental, R., and Scott, E.J.Y., J. Chem. Soc., 1685, (1949).

94. Schoental, R., Nature, 180, 606, (1957).

95. Seelkopt, C.Z., Z. Lebensmitt-Untersuch. Forch., 100, 218, (1955).

96. Smith, W.E., Sunderland, D.A., and Sugiura, K., A.M.A. Arch. Ind. Hyg. and Occup. Med., 4, 299, (1951).

97. Stocks, P., Bri. J. Cancer, 6, 99, (1952).

98. Idem, B.E.C.C. Ann. Rep. (Supplement to Part II) (1957).

99. Tarbell, D.S., Brooker, E.G., Vanterpool, A., Conway,W., Claus C.J. and Hall, T.J., J.A.C.S.,77, 767, (1955).

100. Waller, R.E., Bri. J. Cancer, 6, 8, (1952).

101. Warburg, O., Science, 123, 309, (1956).

102. Weinhouse, S., Adv. in Can. Res., 3, 270, (1955).

103. Wynder, E.L., and Graham, E.A., J. Am.Med.Assoc., 143, 329, (1950).

104. Iidem and Croninger, A.B., Can. Res., 13, 855, (1953).

105. Wynder, E.L., and Wright, G., Cancer, 10, 255, (1957).

106. Austen, D.E.G., Ingram, D.J.E., and Tapley, J.G., Trans. Faraday Soc., 54, Part 3., 400, (1958).